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(54) Title: PURIFICATION OF SERINE PROTEASE AND SYNTHETIC INHIBITORS THEREOF

(57) Abstract

The present invention relates to new compounds of the general formula: 2-Xaa-Y', in which Xaa is an amino acid, Z is a protecting group and Y' is one of various types of ring structures. The new compounds have a modulating activity on serine proteases. The invention further relates to the use of the compounds in therapy and diagnosis and to a method of purifying the serine protease DPPIV.

PURIFICATION OF SERINE PROTEASE AND SYNTHETIC INHIBITORS THEREOF

The present invention lies in the field of serine
5 proteases, in particular serine proteases cleaving a post
proline bond, such as the exopeptidase dipeptidyl peptidase
IV (DPPIV) and the endopeptidase prolyl oligopeptidase (PO
or PEP).

The invention relates to a novel method to purify
10 dipeptidyl peptidase IV (DPPIV) from mammalian, specifically
human, origin and to its therapeutic and diagnostic use. The
invention also relates to a new source for the isolation of
prolyl oligopeptidase (PEP) and to its therapeutic and
diagnostic use. Furthermore, the invention relates to
15 synthetic inhibitors of PEP and dipeptidyl peptidase IV,
methods of their preparation and their therapeutic and
diagnostic use.

Many biological important peptide sequences
contain proline. It confers unique conformational
20 constraints on the peptide chain because the α-nitrogen atom
of proline is part of the rigid pyrrolidine ring and, at the
same time, is covalently bound by means of a secondary amide
bond to the N-terminal preceding amino acid. Thus, the side-
chain is cyclized back on to the backbone amide position.
25 Inside an α-helix the possibility of making hydrogen bonds
to the preceding turn is thereby lost and a kink will be
introduced in the helix. The conformational restrictions
imposed by proline motifs in a peptide chain probably
implicate important structural or biological functions since
30 a high degree of conservation is found in many proteins and
peptides.

An endo or C-terminal Pro-Pro bond and an endo
pre-Pro peptide bond possess a high degree of resistance to
any mammalian proteolytic enzyme. Only a limited number of
35 peptidases are known to be able to hydrolyse proline
adjacent bonds. Their activity is restricted by the isomeric
state and the position of proline in the peptide chain.

Dipeptidyl peptidase IV (DPPIV, also commonly referred to as CD26) is one of these peptidases. It is a serine protease which specifically cleaves N-terminal dipeptides from proteins and polypeptides carrying an
5 unsubstituted N-terminus and a penultimate proline (or alanine) residue in trans conformation. DPPIV is a homodimer with subunit molecular weight of 110 kDa. It is a membrane protein expressed on the surface of lymphoid cells, epithelial and endothelial cells. Surface expression is
10 increased significantly by T-cell activation. DPPIV has been reported to play a role in numerous processes such as T-cell costimulation, binding to proteins of the extracellular matrix, attachment of cancer cells to endothelium, binding to plasma adenosine deaminase, cooperation with entry of
15 human immunodeficiency virus (HIV) into lymphoid cells.

The proteolytic activity of DPPIV resides in a stretch of approximately 200 amino acids located at the C-terminal end of the protein. The catalytic residues (Ser-629, Asp-708, His-740) are arranged in a unique order which
20 is different from the classical serine proteases such as chymotrypsin and subtilisin. Proline specific dipeptidyl peptidase activity alters the biological activity of a large number of bioactive proteins and polypeptides comprising, amongst others, the neurotransmitter substance P, human
25 growth hormone-releasing factor, erythropoietin, interleukin 2 and many others. Since many of these peptides have important effector functions, abnormal DPPIV activity - either too low or too high - will be reflected in an abnormal biological effect. Potential DPPIV substrates are
30 listed in tables 1 and 2. The skilled person will easily recognize that interference with the effector function of these polypeptides may result in clinical conditions including inflammation, vascular diseases, auto-immune disease, multiple sclerosis, joint diseases and diseases
35 associated with benign and malign cell transformation.

Table 1
Human cytokines, growth factors, neuro- and vasoactive peptides
with a penultimate proline,which are putative substrates for DPP IV

Polypeptide	N-terminal sequence
Interleukin-1β	Ala-Pro-Val-Arg-Ser-
Interleukin-2	Ala-Pro-Thr-Ser-Ser-
Interleukin-5	Ile-Pro-Thr-Glu-Ile-
Interleukin-6	Val-Pro-Pro-Gly-Glu-
Interleukin-10	Ser-Pro-Gly-Gln-Gly-
Interleukin-13 (recombinant)	Ser-Pro-Gly-Pro-Val-
Complement C4a	Lys-Pro-Arg-Leu-Leu-
Granulocyte chemotactic protein II	Gly-Pro-Val-Ser-Ala-
Granulocyte macrophage colony stimulating factor	Ala-Pro-Ala-Arg-Ser-
Granulocyte colony stimulating factor.	Thr-Pro-Leu-Gly-Pro
Erythropoietin	Ala-Pro-Pro-Arg-Leu-
Gastrin releasing peptide	
Growth hormone	Phe-Pro-Thr-Ile-Pro-
Interferon inducible peptide 10 (γIP10)	Val-Pro-Leu-Ser-Arg-
Interferon regulatory factor 1 (IRF-1)	
Interferon regulatory factor 2 (IRF-2)	
Insulin-like growth factor-1	Gly-Pro-Glu-Thr-Leu-
Melanoma growth stimulating activity	Ala-Pro-Leu-Ala-Thr-
Migration inhibition factor	Met-Pro-Met-Phe-Ile-
Monocyte chemotactic protein I	Glu-Pro-Asp-Ala-Ile-
Neuropeptide Y	Tyr-Pro-Ser-Lys-Pro-
Pancreatic polypeptide	Ala-Pro-Leu-Glu-Pro-
Peptide YY	Tyr-Pro-Ile-Lys-Pro-
Prolactin	Leu-Pro-Ile-Cys-Pro-
RANTES	Ser-Pro-Tyr-Ser-Ser-
Substance P *	Arg-Pro-Lys-Pro-Gln-
Thrombopoietin	Ser-Pro-Ala-Pro-Pro-
Transforming protein (N-myc) version 1	Met-Pro-Gly-Met-Ile-
Transforming protein (N-myc) version 2	Met-Pro-Ser-Cys-Ser-
Tumor necrosis factor β	Leu-Pro-Gly-Val-Leu-
Vascular endothelial growth factor	Ala-Pro-Met-Ala-Glu-

* hydrolysis by DPP IV has already been proven (4,5).

Table 2

Human peptides and proteins with a penultimate alanine,

which are putative substrates for DPP IV

Polypeptide

---

adenosine deaminase
annexins
breast basic conserved protein
cofilin
natural killer cell enhancing factor b
precursors of interferon α
precursors of interleukin 1-α and β, and interleukin 13
precursors of macrophage inflammatory protein-2 -α- and β
precursor of melanocyte stimulating hormone
precursor of oxytocin-neurophysin 1
Growth hormone releasing hormone *
β amyloid protein (1-28)
Anxiety peptide
'joining peptide' of pro-opiomelanocortin

* hydrolysis by DPP IV has already been proven (6).

5

DPPIV activity in serum is decreased in patients with auto-immune diseases and in patients receiving immunosuppressant drugs (Hagihara, M., Ohhashi, M., Nagatsu, T. (1987) Clin. Chem. 33, 1463-1465; Doctoral thesis I. De
5 Meester, 1992, "Characterization of Human Lymphocytic Dipeptidyl Peptidase IV and its identification as the Activation Antigen CD26", Universitaire Instelling Antwerpen, Departement Farmaceutische Wetenschappen.

Examples of auto-immune diseases are rheumatoid
10 arthritis, systemic lupus erythematosus (this is a vascular disease), multiple sclerosis, Serum DPPIV activity is also decreased in malignancies such as gastrointestinal cancers (Haacke, W., Kullerts, G., & Barth, A. (1986) Arch. Geschwulstforsch 56, 145-153) and blood cancers including
15 acute lymphoid leukemia, lymphosarcoma and Hodgkin's disease (Kojima, J., Ueno, Y., Kasugai, H., Okuda, S., & Akedo, H. (1987) Clin. Chim. Acta 167, 285-291; Fujita, K., Hirano, M., Tokunaga, K., Nagatsu, T., & Sakakibara, S. (1977>) Clin. Chim. Acta 81, 215-217). DPPIV activity is increased
20 in patients with hepatobiliary disease and hepatic cancer (Burchardt, U., Klagge, M., Hafstein, M., Salka, S., Graupner, H., Lenski, K., Delev, B., Neubert, K. & Barth, A. (1987) Z. Klin. Med. 42, 245-249). A role in inflammation can also be inferred from the observation that DPPIV is
25 involved in costimulation and proliferation of granulocytes and macrophages (Bristol, L.A., Sakaguchi, K., Appella, E., Doyle, D., & Takacs, L. (1992) J. Immunol. 149, 367-372).

Also the response of T-lymphocytes to recall antigen and the entry of HIV have been reported to be at
30 least partially influenced by the catalytic site of DPPIV. Recall antigen can be defined as an antigen that activates the immunological memory. It is an antigen to which the individual has been exposed before and which has previously triggered an immunological response. Tanaka et al. (Proc.
35 Natl. Acad. Sci. USA 90, 4586-4590 (1993)) demonstrated that the active site of DPPIV is involved in the response to recall antigen. Callebaut et al. (Science 262, 2045-2046

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(1993)) found an effect of catalytic site ligands in their model of HIV entry.

Prolyl endopeptidase (PEP), also called proline oligopeptidase (PO), was first discovered by Walter and
5 coworkers as an oxytocin-degrading enzyme in the human uterus (Walter et al., Science 173, 827-829 (1971)). The enzyme cleaves peptide bonds at the carboxy-side of proline in peptides containing the sequence X-Pro-Y, where X is a peptide or N-terminal substituted amino-acid and Y is a
10 peptide, amino acid, amide or alcohol (Yoshimoto et al., J. Biol. Chem. 253, 3708-3716 (1979)). The enzyme has a high specificity for the _trans_-conformation of the peptide bond at the imino-side of proline (Lin & Brandts, Biochemistry 22, 4480-4485 (1983)).

15 Prolyl endopeptidase has been characterized and purified form plants, micro-organisms and various mammalian sources. The enzyme is widely distributed among different mammalian tissues and high activity is found in brain, kidney, lung and muscle. The human enzyme has been purified
20 from lung, brain, erythrocytes and placenta (Kalwant & Porter, Biochem. J. 276, 237-244 (1992)).

The sequences of prolyl oligopeptidase from _A._ _hydrophila_ (Kanatani et al., J. Biochem. 113, 790-796 (1993)), _F._ _meningosepticum_ (Yoshimoto et al., Agric. Biol.
25 Chem. 55, 37-43 (1991)), porcine brain (Rennex et al., Biochemistry 30, 2195-2203 (1991)) and human lymphocytes (Vanhoof et al., Gene 149, 363-366 (1994)) have been determined.

In vitro PEP catalyzes the cleavage of several
30 biologically active peptides such as angiotensin II, oxytocin, vasopressin, bradykinin. _In vivo_ however its function remains obscure (Welches et al., Life Sci. 52, 1461-1480 (1993)). Recently it was shown that the prolyl oligopeptidase activity in plasma correlates well with the
35 different stages of depression. Maes et al. (Biological Psychiatry 30, 577-586 (1991)) showed that DPPIV is decreased in major depression. It was proven to be the best biochemical marker for depression found so far.

7

Hydrolysis of angiotensin I and angiotensin II by prolyl oligopeptidase results in the liberation of angiotensin (1-7). Angiotensin (1-7) has vasodilator activity and modulates the release of vasopressin, which is

5 able to influence the process of memory as was shown by injecting rats with specific PEP-inhibitors. The injection reverses the scopolamine induced amnesia. This experiment is not only an example which provides evidence for a possible physiologic function for the enzyme, but moreover it has led

10 to the hypothesis that inhibitors for PEP can influence the memory process and counter dementia (Yoshimoto et al., J. Pharmacobio-Dyn. 10, 730-735 (1987); Furukawa et al. , J. Pharmacobio-Dyn 12, S136 (1989)).

PEP may also be involved in the regulation of the
15 blood pressure by participating in the renin-angiotensin system. PEP can decrease the amount of angiotensin II and its precursor and moreover the peptide generated by cleavage by PEP, angiotensin (1-7) itself has vasodilator activity (Kohara et al., Hypertension 17, 131-138 (1991)).

20 Furthermore, involvement of PEP in vascular disease can be inferred from its ability to cleave bradykinin (which controls blood pressure) and blood coagulation factor X.

As indicated above, the proline specific serine proteases probably have a role in various physiological

25 processes in the human and animal body, which processes might be related to various disease states, both physical and mental.

It is an object of the invention to interfere in the physiological action of proline specific serine

30 proteases, such as PEP and DPPIV, to be able to treat the said disease states.

It is a further object of the invention to provide a tool for diagnosing such disease states.

According to a first aspect the invention

35 therefore provides compounds according to claim 1, which compounds are able to inhibit the enzymatic activity of serine proteases, such as PEP and DPPIV.

According to a second aspect the invention provides a method for isolating DPPIV and a method for the isolation of PEP from a source not previously used.

The modulating compounds and the isolated enzymes

5 may be used in various therapeutical and diagnostic applications as follows.

The modulators (inhibitors) may be used in pharmacotherapy of disease states related to inflammation, dysfunction of the immune system, vascular disease and

10 proliferation disorders, for artificial induction of a state of immunosuppression, or in pharmacotherapy of neurological diseases. Diagnostic applications relate to imaging (wherein the inhibitors may be optionally labelled) and _in vitro_ and _in vivo_ diagnostic function tests.

15   The isolated enzyme may for example be used to enhance the activity of the endogenous enzyme, for example in immune stimulation as further described hereinbelow.

Hereinbelow the invention will be described in more detail. The Examples disclose the synthesis of the

20 modulating compounds of the invention, the inhibitory activity of the compounds of the invention on the catalytic activity of human dipeptidyl peptidase IV (CD26) and PEP and the new isolation method of DPPIV and PEP.

In this application the terms "inhibitor",

25 "compound", "derivative" and "modulating compound" are used interchangeably. Although the compounds will mostly have inhibiting properties they may in some situations have enhancing properties.

The synthetic inhibitors of the present invention

30 are derived from dipeptides. The carboxylate group of the C-terminal amino acid is replaced by a hydrogen atom or a functional group mimicking the transition state of the enzyme or capable of specific modification of the active site. The C-terminal amino acid is proline, homoproline,

35 azetidine, hydroxyproline or another substituted proline homolog. The N-terminal amino acid can be any naturally occurring amino acid or an amino acid which does not occur in nature, preferably with an aliphatic or aromatic side

chain, or proline. Examples of naturally occurring amino
acids with aliphatic or aromatic side chains are leucine,
isoleucine, valine, alanine, phenylalanine, tyrosine,
tryptophan. Examples of amino acids which do not occur in
5 nature are the D-isomers of the compounds mentioned before,
synthetic amino acids with prostethic groups such as
cyclohexylalanine, 1-naphtylalanine, p-fluoro-phenylalanine,
o-tbutyl-tyrosine. Examples of the C-terminal substituent
group are phosphonate esters, phosphinates or a hydrogen
10 atom.

The present invention also includes derivatives
which have been modified in the N-terminal amino acid side
chain without abolishing the reactivity with the active
site. Examples of such modifications are the incorporation
15 of a radioactive label such as Iodine^{125} into tyrosine,
extension of the side chain to attach biotin or a
fluorophore. To improve the half-life in the circulation the
peptide bond between the two amino acids may be replaced by
a non-hydrolyzable bond. The incorporation of a radioactive
20 label is useful in diagnostic methods using the modulating
compounds.

The compounds of the invention may be divided in a
group in which the amino terminus is protected and a group
of compounds which are not protected. The first group is
25 particularly useful for inhibiting the activity of PEP,
whereas the second type of compounds is specific for DPPIV.
The protecting group may be any suitable group and its
selection is within the scope of the skilled person in the
field. A suitable protecting group is the benzyloxycarbonyl
30 (Cbz) group.

Preferred compounds of this invention contain the
diphenyl-phosphonate group (indicated with the symbol
$^P(OPh)_2$). Substituted phenyl esters can also be used, for
example p-chloro-phenyl esters or p-benzoic acid esters and
35 their derivatives. The most preferred compounds of this type
are: Pro-Pro^P-(OPh)$_2$, Phe-Pro^P-(OPh)$_2$, Ile-Pro^P-(OPh)$_2$, O-
tButyl-Tyr-Pro^P-(OPh)$_2$, para-Fluoro-Phe-Pro-^{P}(OPh)$_2$,

cyclohexylalanine-Pro-^{P}(OPh)$_2$, Ala-Pro-^{P}(OPh)$_2$, Arg-Pro^P-
(OPh)$_2$, 1-naphtyl-Ala-Pro^P-(OPh)$_2$.

In another embodiment the compounds comprise a
pyrollidine ring, that may optionally be substituted with a
5 halogen. Of these compounds Ile-3-Fluoro-pyrrolidine is a
particularly active inhibitor for DPPIV and Z-Pro-3-Fluoro-
pyrrolidine for PEP.

A general description of the synthesis of the
phosphonates is given below.

10
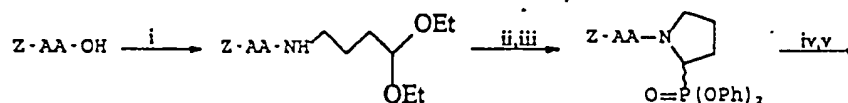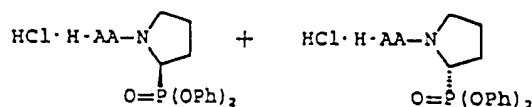
	(S)-AA
a	Pro
b	Phe
c	p-F-Phe
d	cyclohexyl-Ala
e	Ile
f	1-naphthyl-Ala
g	Arg
h	O-t-Bu-Tyr
i	Ala

15

20

i   Method A: i-BuOCOCl, Et$_3$N, 4-ABAD, CHCl$_3$,-10°C, r.t.
        Method B: pentafluorophenol, DCC, 4-ABAD, CHCl$_3$, 0°C,
25   r.t.
ii   0.5N HCl/THF, r.t., 1h.
iii   P(OPh)$_3$, AcOH, 80-85°C, 1 h.
iv   H$_2$/Pd/C, MeOH/AcOH, r.t., 3-4 h.
v   vacuum column chromatography: CHCl$_3$:MeOH:AcOH (25:1:1)
30   0.5N HCl/EtAc

The benzyloxycarbonyl (Cbz- or Z-)protected amino acids were
coupled to 4-aminobutyraldehyde diethyl acetal (4-ABAD)
using the standard mixed anhydride method (Bodanski, M. & A.
35 Bodanski, in: The Practice of Peptide Synthesis, Springer
Verlag, Berlin, p.103-111 (1984)) (isobutylchloroformate,
triethylamine) or through the pentafluorofenol ester (DCC).
The diethyl acetals were hydrolysed with 0.5N HCl in

tetrahydrofuran (THF) (Matsuda F. et al.; Tetrahedron 41,
3625 (1985)) and the resulting mixture was treated with
triphenylphosphite and acetic acid for one hour at 80-85°C.
The cyclization of the acetal to pyrrolidine-2-phosphonate

5 was based upon a method of Oleksyszyn and coworkers,
(Synthesis, 985-986 (1979)). The obtained mixture of
diastereoisomers were separated by vacuum chromatography.
Deprotection of these products was performed by
hydrogenolysis with hydrogen-palladium. Details of the

10 synthesis and physicochemical properties of the compounds
are given in Example 1. The evaluation of the inhibitory
activity of the compounds is the subject of Example 2.

     The ability of DPPIV/CD26 to specifically form a
stable complex with adenosine deaminase was exploited to

15 develop a novel purification method for DPPIV. Adenosine
deaminase is coupled to a suitable matrix, for example
dextran beads, latex, plastic or glass. DPPIV binds to the
matrix. Unbound material is removed and DPPIV is eluted with
a gentle washing procedure. A specific procedure using ADA

20 coupled to cyanogen bromide activated Sepharose 4B is
described in Example 5. The purification protocol is part of
this invention.

     Because of the high specificity of the interaction
between ADA and DPPIV a method such as described in Example

25 5 can also be used to purify DPPIV from complex mixtures
such as biological fluids, tissue extracts or cell culture
extracts used for expression of recombinant forms of DPPIV.
Apart from the naturally occurring mammalian DPPIV,
recombinant full-length protein, mutated forms with altered

30 enzymatic activity, altered glycosylation patterns or
truncated forms may be purified with this method as long as
the ADA binding site remains intact. The same applies for
DPPIV which has been chemically modified, for example linked
to a PEG tail to improve the half life in the circulation,

35 isotopically labelled or linked to a reporter enzyme or a
fluorophore. Protein preparations obtained using this method
are also understood to be part of this invention.

According to the invention it has been found that
PEP may very well be isolated from peripheral blood
mononuclear cells (PBMC) and thrombocytes. Up till now this
source for PEP has not been disclosed. The isolation of PEP
5 from PBMC is the subject of Example 7.

The compounds described above can be used for the
therapy of pathological states associated with excessive,
impaired or unbalanced DPPIV or PEP activity.

Such clinical conditions may for example arise
10 from the role of DPPIV in the function of the immunological
memory, for example in autoimmune disease and foreign tissue
rejection after transplantation (Fleisher, B., Immunology
Today 15, 180-184 (1994); Morimoto, C. & S.F. Schlossman,
The Immunologist 2, 4-7 (1994)). In such cases the DPPIV
15 inhibitors of this invention can be used as immuno-
suppressants.

In other cases, such as infections, an increased
immune response may be required which can be modulated by
administration of purified DPPIV preparations (Tanaka, T. et
20 al., Proc. Natl. Acad. Sci. USA 91, 3082-3086 (1994)).

Improper processing or inactivation of biological
effector molecules carrying the N-terminal X-Pro or X-Ala
motif (see Tables 1 and 2) may imply DPPIV activity in a
range of clinical conditions comprising, for example,
25 inflammation, vascular disease, autoimmune disease, multiple
sclerosis, joint diseases, diseases associated with benign
and malign cell transformations.

Starting from the available information on the
correlation between DPPIV or PEP and various disease states
30 the skilled person will be able to define therapeutical
utilities for the inhibitory compounds of the invention.

The invention also relates to the diagnostic use
of the compounds. Labeled inhibitors can be used essentially
in the same type of applications as labeled monoclonal
35 antibodies, e.g. fluorescence and radioassays,
cytofluorimetry, fluorescence activated cell sorting etc..
The principles of such techniques can be found in
immunochemistry handbooks, for example: A Johnstone and R.

13

Thorpe, Immunochemistry in practice, 2nd. Ed. (1987), blackwell Scientific publications, Oxford London Edinburgh Boston Palo Alto Melbourne.

5   In cytochemistry and histochemistry labeled inhibitors can be used to directly visualize the cellular distribution of the target protease (DPPIV or PEP). The label can be fluorescent for fluorescence microscopy, radioactive for autoradiography, or electron dense for electron microscopy. The target structures can be whole

10 cells, cells fixed onto slides or sections through solid tissue. A useful modification of these techniques is to use an indirect ("sandwich") assay employing the specific high affinity interaction between biotin and avidin (reviewed in Methods in Enzymology, vol. 184, 1990).

15   For imaging of tumours expressing high amounts of the target protease (DPPIV or PEP), inhibitors labeled with a suitable isotope (e.g. ^{123}I or ^{131}I) can be injected and after clearing of the excess inhibitor from the circulation, the tumour can be visualized by radioscintigraphy. The

20 principles of imaging are summarized by A. Bamias & A.A. Epenetos (1995) in: Monoclonal antibodies, production, engineering and clinical application (M.A. Ritter and M.M. Ladyman, eds.) Cambridge university press, Cambridge New York Melbourne, pp. 222-246.

25   Localisation of DPPIV by means of monoclonal antibody and by enzymatic activity was reported to be diagnostically useful and applicable to clinical material in the case of thyroid follicular tumours and thyroid papillary carcinoma (Kotani, T et al., Int. J. Exp. Path. 73, 215-222

30 (1992); Kotani, T. et al., J. Path. 168, 41-45 (1992)). In an analogous manner the compounds of the invention which form a stable adduct with DPPIV may be used as a tool for diagnosing certain disease states.

It has been shown that DPPIV expression is

35 specifically downregulated during malignant transformation of melanocytes. DPPIV is expressed on the surface of normal melanocytes but not on malignant melanoma cells (Morrison, M.E., Vijayaradhi, S., Engelstein, D., Albino, A.P. &

Houghton, A.N. (1993) J. Exp. Med. 177, 1135-1143).
According to the invention specific cytochemical or
histochemical localisation of DPPIV can be used to diagnose
malignancy in melanoma cells.

5   The present invention furthermore relates to the
use of the inhibitory compounds in various therapeutical
applications. In order to demonstrate the usefulness of
these compounds in therapy some experiments have been
performed showing <u>in vivo</u> inhibitory activity of the
10 compounds on both DPPIV and PEP. Example 4 shows the DPPIV
catalyzed degradation of vasostatin and inhibition thereof,
whereas Example 9 relates to the activity of PEP on
bradykinin and substance P, a neurotransmitter peptide with
an aminoterminal X-Pro-motif and the inhibition thereof as a
15 model for the potential therapeutic use of the inhibitors in
medicin.

       The present invention thus also encompasses
pharmaceutical compositions prepared for storage and
subsequent administration, which have a pharmaceutically
20 effective amount of the compounds disclosed above in a
pharmaceutically acceptable carrier or diluent. Acceptable
carriers and diluents are well known and are described, for
example, in Remington's Pharmaceutical Sciences, Mack
Publishing Co. (A. R. Gennaro edit. 1985). Preservatives,
25 stabilizers, dyes and flavoring agents may be provided in
the pharmaceutical compositions. For example, sodium
benzoate, sorbic acid and esters of p-hydroxybenzoic acid
may be added as preservatives. In addition antioxidants and
suspending agents may be used.

30   The compositions of this invention may be
formulated and used as tablets, capsules or elixirs for oral
administration; suppositories for rectal administration;
sterile solutions or suspensions for injectable
administration; aerosols; unguents for topical
35 administration. If desired, absorption enhancing
preparations (e.g. liposomes) or other appropriate delivery
systems may be used. The amount of the active substance(s)
in a dosage unit may vary between 0.01 mg and 1 g.

15

The compounds or compositions of this invention can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. The dosage for the compounds of the present invention can range

5 broadly depending upon the desired effects and the therapeutic indication.

The DPPIV inhibitors described above which form covalent, long-lived adducts with DPPIV are therefore suitable for diagnostic applications such as imaging and

10 histochemical staining of DPPIV. This requires the introduction of a radio-isotope, e.g. iodine, or a fluorescent or other type of reporter group. Because of their small size they are expected to penetrate tissue more easily as, for example, antibodies. Moreover it was found

15 that they only recognize DPPIV which is native and enzymatically active. Formulations of the compounds to be used in diagnostic applications are also part of this invention.

DPPIV activity can interfere with other enzymatic

20 assays by cleaving the substrate used in the test and thereby giving either false positive (when a chromogenic substrate is cleaved) or false negative results (when a peptide substrate is degraded). The inhibitors of this invention can be used to inactivate contaminating DPPIV

25 before carrying on with the analysis. An example of this is the fluorogenic assay for proline specific endoprotease the substrate of which, Gly-Ala-4-Me-2-NA, is also cleaved by DPPIV. Another application can be found in the decontamination of aminopeptidase M preparations.

30   The DPPIV inhibitors can also be used to avoid degradation of bioactive peptides which contain an aminoterminal X-Pro-motif (e.g. cytokines) in biological samples prior to analysis.

The present invention will be further elucidated

35 with reference to the following examples which are only given for illustration purposes and are in no way intended to limit the invention.

EXAMPLES

EXAMPLE 1

Synthesis and characterization of modulating compounds of the invention

5 **A. PHOSPONATES**

a) Materials and methods

The amino acids used in the synthesis were obtained from various manufacturers. (S)-alanine, (S)-isoleucine, (S)-phenylalanine, (S)-proline were purchased

10 from Janssen Chimica NV; tri-Z-(S)-arginine, (S)-homoproline, Z-(S)-p-fluorophenylalanine, (S)-cyclohexylalanine, (S)-(1-naphtyl)alanine, (S)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid [(S)-Tic] were purchased from Bachem AG.

15 Melting points were determined on a Digital Melting Point Apparatus (Electrothermal) and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker (300 MHz) spectrometer. Observed rotations at the Na-D line were obtained at 20°C using a Perkin-Elmer 241 polarimeter.

20 Mass-spectra were recorded on a VG 70 SEQ (Fisons, VG analytical) spectrometer. TLC was carried out on plates precoated with silica gel Merck UV-254. Column chromatography was performed with silica gel H, 5-40 μm, purchased from Fluka, Aldrich, Sigma.

25

b) Synthesis of the intermediate (4-[(S)-2-benzyloxy-carbonylaminoacyl] aminobutyraldehyde diethyl acetals

Acetals are intermediates in the synthesis of were prepared by coupling the corresponding N-

30 benzyloxycarbonyl (N-Cbz) protected amino acid with 4-aminobutyraldehyde diethyl acetal using the standard mixed anhydride method (supra) with isobutylchloroformate (Method A) or via pentafluorophenol ester without isolation of the latter (Method B). The following intermediates were

35 prepared:

1.   4-(N-Cbz-(S)-alanyl)aminobutyraldehyde diethyl acetal: yield 65% (Method A), oil.

2.   4-(N-Cbz-(S)-isoleucyl)aminobutyraldehyde diethyl
      acetal:
      yield 77% (Method A), mp 135°C, $[\alpha]_D^{20}$= -5.5° (C1,
      CHCl$_3$).

5   3.   4-(N-Cbz-(S)-phenylalanyl)aminobutyraldehyde diethyl
          acetal:
          yield 66% (Method A), mp 145°C, $[\alpha]_D^{20}$= 6.0° (C1, CHCl$_3$).

    4.   4-(N^2,N^7,N$^{7'}$-tri-Cbz-(S)-arginyl)aminobutyraldehyde
          diethyl acetal:

10   yield 99% (Method B), mp 158°C, $[\alpha]_D^{20}$= 12.1° (C1,
          CHCl$_3$).

    5.   4-(N-Cbz-(S)-prolyl)aminobutyraldehyde diethyl acetal:
          yield 92% (Method B, purified by the vacuum column
          chromatography), mp 67°C, $[\alpha]_D^{20}$= -68.7° (C1, CHCl$_3$).

15 6.   4-(N-Cbz-(S)-homoprolyl)aminobutyraldehyde diethyl
          acetal:
          yield 93% (Method B, purified by the vacuum column
          chromatography), oil, $[\alpha]_D^{20}$= -48.4° (C1, CHCl$_3$).

    7.   4-(N-Cbz-(S)-p-fluorophenylalanyl)aminobutyraldehyde
20   diethyl acetal:
          yield 99% (Method B), mp 143°C, $[\alpha]_D^{20}$= 2.8° (C1, CHCl$_3$).

    8.   4-[N-Cbz-(S)-(1-naphtyl)alanyl]aminobutyraldehyde.
          diethyl acetal:
          yield 81% (Method B, purified by the vacuum column
25   chromatography), mp 112 °C.

    9.   4-(N-Cbz-(S)-cyclohexylalanyl)aminobutyraldehyde
          diethyl acetal:
          yield 81% (Method B, purified by the vacuum column
          chromatography), mp 102°C, $[\alpha]_D^{20}$= -13.3° (C1, CHCl$_3$).

30 10.   4-[(N-Cbz-(S)-tetrahydroisoquinoline)-3-
          carbonyl]aminobutyraldehyde diethyl acetal:
          yield 75 % (Method B, purified by the vacuum column
          chromatography), oil, $[\alpha]_D^{20}$= -2.1° (C1, CHCl$_3$).

35 c)   Synthesis of diphenyl 1-[(S)-2-benzyloxycarbonyl-
         amino-acyl] pyrrolidin-2-phosphonates (General
         procedure for protected phosphonates)

0.5 N aqueous HCl solution (30 ml, 15 mmole) was
added at room temperature to the solution of diethyl acetal
(10 mmole) in THF (60 ml). The mixture was stirred for 1 h.
After the addition of ether (85 ml), the pH of the mixture
5 was adjusted to 7-8 with 1N NaOH. The organic layer was
separated and the aqueous layer was washed with ether (60
ml). The combined organic layer was dried (MgSO$_4$) and
evaporated to dryness under reduced pressure. The resulting
oil was dissolved in acetic acid (30 ml), triphenylphosphite
10 [P(OPh)$_3$] (20 mmole) was added and the solution was stirred
at 80-85°C for 1 h. The mixture was cooled and evaporated at
45-50 °C under reduced pressure. The residue was dissolved
in chloroform (50 ml). The solution was washed with water,
saturated NaHCO$_3$ solution, dried (MgSO$_4$) and evaporated to
15 dryness under reduced pressure. The crude compound was
purified on a vacuum column using ethyl acetate - petroleum
ether (1:1 or 1:2) as mobile phase. The following compounds
were prepared:

20 11.   Diphenyl 1-(N-Cbz-(S)-alanyl)pyrrolidine-2-phosphonate:
         yield 65%, oil. ^{1}H-NMR (CDCl$_3$/TMS): 1.35 (dd, 3H), 1.8-
         2.7 (m, 4H), 3.3-3.9 (m, 2H), 4.1 (m, 1H), 4.45 (m,
         1H), 5.1 (m, 2H), 5.8 (m, 1H), 7.0-7.4 (m, 15H).

   12.   Diphenyl 1-(N-Cbz-(S)-isoleucyl)pyrrolidine-2-
25   phosphonate:
         yield 66%, oil. ^{1}H-NMR (CDCl$_3$/TMS): 0.8 (t, 3H), 1.0
         (d, 3H), 1.25 (m, 1H), 1.55 (m, 1H), 1.9-2.8 (m, 4H),
         3.4-3.9 (m, 2H), 4.4 (m, 1H), 5.1 (m, 2H), 5.5 (m, 2H),
         7.0-7.4 (m, 15H).

30 13.   Diphenyl 1-(N-Cbz-(S)-phenylalanyl)pyrrolidine-2-
         phosphonate:
         yield 74%, semicrystalline oil. ^{1}H-NMR (CDCl$_3$/TMS):
         1.3-2.7 (m, 4H), 2.8-3.6 (m, 4H), 4.7 (m, 1H), 5.0 (m,
         1H), 5.1 (m, 2H), 5.35 (m, 1H), 7.0-7.4 (m, 20H). MS
35   (FAB) m/e (rel. intensity): 585 (M+H)$^+$ (100).

   14.   Diphenyl 1-(N^2,N^7,N$^{7'}$-tri-Cbz-(S)-arginyl)pyrrolidine-2-
         phosphonate:
         yield 40%, oil. ^{1}H-NMR (CDCl$_3$/TMS):

15. Diphenyl 1-(N-Cbz-(S)-prolyl)pyrrolidine-2-phosphonate:
    yield 66%, oil. ^{1}H-NMR (CDCl$_3$/TMS): 1.5-2.8 (m, 8H),
    3.2-4.0 (m, 4H), 4.6 (m, 1H), 4.85 (m, 1H), 5.1 (m,
    2H), 7.0-7.5 (m, 15H).

16. Diphenyl 1-(N-Cbz-(S)-homoprolyl)pyrrolidine-2-
    phosphonate:
    yield 54%, semicrystalline oil. ^{1}H-NMR (CDCl$_3$/TMS):

17. Diphenyl 1-(N-Cbz-(S)-p-fluorophenylalanyl)pyrrolidine-
    2-phosphonate:
    yield 70%, oil. ^{1}H-NMR (CDCl$_3$/TMS): 1.2-2.8 (m, 4H),
    2.9-4.0 (m, 4H), 4.6 (m, 1H), 4.8 (m, 1H), 5.05 (m,
    2H), 5.6 (m, 1H), 6.5-7.5 (m, 19H).

18. Diphenyl 1-(N-Cbz-(S)-cyclohexylalanyl)pyrrolidine-2-
    phosphonate:
    yield 55%, semi-crystalline oil. ^{1}H-NMR (CDCl$_3$/TMS):
    0.6-2.5 (m, 17H), 3.75 (m, 2H), 4.6 (m, 1H), 5.0 (m,
    3H), 5.35 (m, 1H), 7.0-7.4 (m, 15 H).

19. Diphenyl 1-[N-Cbz-(S)-(1-naphtyl)alanyl]pyrrolidine-2-
    phosphonate:
    yield 68%, semicrystalline oil. ^{1}H-NMR (CDCl$_3$/TMS):
    1.0-2.4 (m, 4H), 2.8-4.0 (m, 4H), 5.1 (m, 3H), 5.35 (m,
    1H), 6.05 (m, 1H), 6.9-8.5 (m, 22H).

20. Diphenyl 1-[(N-Cbz-(S)-tetrahydroisoquinolyl)-3-
    carbonyl]pyrrolidine-2-phosphonate:
    yield 76%, semicrystalline oil. ^{1}H-NMR (CDCl$_3$/TMS):
    1.5-2.5 (m, 4H), 2.9 (m, 3H), 3.45 (m, 2H), 3.75 (m,
    1H), 4.0 (m, 1H), 4.5-5.3 (m, 3H), 6.9-7.5 (m, 19H).

d) Diphenyl (S)- and (R)-1-[(S)-aminoacyl]pyrrolidine-2-
phosphonate hydrochlorides (General procedure for
unprotected phosponates).

Method A

        Z(or Cbz)-protected phosphonate (1 mmole) was
dissolved in a mixture of methanol (20 ml) and acetic acid
(0.5 ml). The solution was hydrogenated over 10% Pd/C at
room temperature for 3-6 h (the reaction was monitored by
TLC). The catalyst was removed on a celite filter and the

20

filtrate was evaporated under reduced pressure. The residue
was dissolved in dry $CHCl_3$, 0.5N HCl in EtOAc (2.5 ml) was
added and the product was precipitated with ether or hexane-
ether mixtures. The solid was collected and dried in a
5 vacuum desiccator over NaOH. This procedure has been used
when no separation of diastereomers could be detected on
TLC.

Method B

10   Z-Protected phosphonate was deblocked as above,
but after removal of the catalyst and evaporation of the
filtrate the residue was purified on a vacuum column with
$CHCl_3$-MeOH-AcOH (25:1:1) as eluent. Fractions containing
pure diastereomers were evaporated under reduced pressure,
15 the residue was dissolved in dry $CHCl_3$, 0.5N HCl in EtOAc
was added and the product was precipitated with ether or
hexane-ether mixtures. The solid was collected and dried in
the vacuum desiccator over NaOH.

        Most of dipeptide phosphonate hydrochlorides obtained
20 melted in a wide interval and (or) with decomposition and
had no clear melting point.

   21. Diphenyl (S,R)-1-[(S)-alanyl]pyrrolidine-2-phosphonate
        hydrochloride:
        yield 60% (method A), hygroscopic solid.
25 22. Diphenyl (R)-1-[(S)-isoleucinyl]pyrrolidine-2-
        phosphonate hydrochloride:
        yield 30% (method B), mp 199°C, $[\alpha]_D^{20}= -26.0°$ (C1,
        MeOH). ^{1}H-NMR ($CDCl_3$/TMS): 0.75 (t, 3H), 1.0 (d, 3H),
        1.25 (m, 1H), 1.55 (m, 1H), 1.8-2.7 (m, 5H), 3.3-4.0
30   (m, 2H), 4.05 (m, 1H), 5.05 (m, 1H), 7.0-7.4 (m, 10H),
        8.35 (br s, 3H). MS (FAB) m/e (rel. intensity): 417 (M-
        Cl)$^+$ (100).
   23. Diphenyl (S,R)-1-[(S)-isoleucinyl]pyrrolidine-2-
        phosphonate hydrochloride:
35   yield 90% (method A), hygroscopic solid.

24. **Diphenyl (S)-1-[(S)-phenylalanyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 37% (method B), hygroscopic solid, $[\alpha]_D^{20} = 103.3°$
   (C1, MeOH). ^{1}H-NMR (CDCl$_3$/TMS): 0.7-2.6 (m, 4H), 2.9-
   3.7 (m, 4H), 3.9 (m, 0.2H), 4.5 (m, 0.8H), 4.7 (m,
   0.8H), 5.25 (m, 0.2H), 6.9-7.5 (m, 15H), 8.2 (br s,
   0.6H), 8.8 (br s, 2.4H). MS (FAB) m/e (rel. intensity):
   451 (M-Cl)$^+$ (100).

25. **Diphenyl (R)-1-[(S)-phenylalanyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 25% (method B), hygroscopic solid, $[\alpha]_D^{20} = 5.5°$
   (C1, MeOH). ^{1}H-NMR (CDCl$_3$/TMS): 1.7-2.6 (m, 4H), 3.1-
   3.8 (m, 4H), 4.4 (m, 1H), 5.05 (m, 1H), 6.8-7.5 (m,
   15H), 8.7 (br s, 3H). MS (FAB) m/e (rel. intensity):
   451 (M-Cl)$^+$ (100).

26. **Diphenyl (S,R)-1-[(S)-arginyl]pyrrolidine-2-phosphonate dihydrochloride:**

   yield 60% (method A), hygroscopic solid.

27. **Diphenyl (S,R)-1-[(S)-prolyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 79% (method A), hygroscopic solid. ^{1}H-NMR
   (CDCl$_3$/TMS): 1.5-2.7 (m, 8H), 3.0-4.0 (m, 4H), 6.3 (m,
   1H), 7.9-8.5 (m, 1H).

28. **Diphenyl (S)-1-[(S)-homoprolyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 43% (method B), hygroscopic solid, $[\alpha]_D^{20} = 48.3°$
   (C1, CHCl$_3$).

29. **Diphenyl (R)-1-[(S)-homoprolyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 40% (method B), hygroscopic solid, $[\alpha]_D^{20} = -45.3°$
   (C1, CHCl$_3$).

30. **Diphenyl (S)-1-[(S)-p-fluorophenylalanyl]pyrrolidine-2-phosphonate hydrochloride:**

   yield 20% (method B), hygroscopic solid, $[\alpha]_D^{20} = 86.6°$
   (C1, MeOH). ^{1}H-NMR (CDCl$_3$/TMS): 0.8-2.7 (m, 4H), 2.8-
   3.7 (m, 4H), 3.9 (m, 0.25H), 4.55 (m, 0.75H), 4.7 (m,
   0.75H), 5.25 (m, 0.25H), 6.5-7.5 (m, 14H), 8.3 (br s,
   0.75H), 8.85 (br s, 2.25H).

22

31. Diphenyl (R)-1-[(S)-p-fluorophenylalanyl]pyrrolidine-2-phosphonate hydrochloride:
yield 16% (method B), hygroscopic solid, $[\alpha]_D^{20} = -2.2°$ (Cl, MeOH). ^{1}H-NMR (CDCl$_3$-CD$_3$OD /TMS): 1.9-2.6 (m, 4H), 2.8-3.2 (m, 2H), 3.4-4.0 (m, 2H), 4.5 (m, 1H), 5.0 (m, 1H), 6.7-7.6 (m, 14H).

32. Diphenyl (S)-1-[(S)-cyclohexylalanyl]pyrrolidine-2-phosphonate hydrochloride:
yield 47% (method B), hygroscopic solid, $[\alpha]_D^{20} = 61.5°$ (Cl, CHCl$_3$). ^{1}H-NMR (CDCl$_3$/TMS): 0.5-2.7 (m, 17H), 3.2-4.1 (m, 2H), 4.1-4.6 (m, 1H), 4.2-5.3 (m, 1H), 6.7-7.5 (m, 10H), 8.36 (br s, 1H), 8.55 (br s, 2H).

33. Diphenyl (R)-1-[(S)-cyclohexylalanyl]pyrrolidine-2-phosphonate hydrochloride:
yield 31% (method B), hygroscopic solid, $[\alpha]_D^{20} = -43.5°$ (Cl, CHCl$_3$). ^{1}H-NMR (CDCl$_3$/TMS): 0.5-3.0 (m, 17H), 3.1-4.1 (m, 2H), 4.3 (m, 1H), 5.35 (m, 1H), 6.7-7.5 (m, 10H), 8.5 (br s, 3H).

34. Diphenyl (S)-1-[(S)-(1-naphtyl)alanyl]pyrrolidine-2-phosphonate hydrochloride:
yield 20% (method B), hygroscopic solid, $[\alpha]_D^{20} = 80.0°$ (C2, CHCl$_3$). ^{1}H-NMR (CDCl$_3$/TMS): 0.5-2.1 (m, 4H), 3.15 (m, 2H), 3.4-4.1 (m, 2H), 4.4-4.6 (m, 2H), 6.7-8.2 (m, 17H), 9.05 (br s, 3H).

35. Diphenyl (R)-1-[(S)-(1-naphtyl)alanyl]pyrrolidine-2-phosphonate hydrochloride:
yield 20% (method B), hygroscopic solid, $[\alpha]_D^{20} = 15.5°$ (C0.5, CHCl$_3$-MeOH 1:1). ^{1}H-NMR (CDCl$_3$-CD$_3$OD/TMS): 0.6-2.5 (m, 4H), 2.7-3.1 (m, 2H), 3.2-4.0 (m, 2H), 4.8-5.0 (m, 2H), 6.7-8.3 (m, 17H).

36. Diphenyl (S)-1-[(S)-(tetrahydroisoquinolyl)-3-carbonyl]pyrrolidine-2-phosphonate hydro-chloride:
yield 24% (method B), hygroscopic solid, $[\alpha]_D^{20} = 51.8°$ (Cl, CHCl$_3$). ^{1}H-NMR (CDCl$_3$/TMS): 1.8-2.8 (m, 4H), 3.1-3.5 (m, 3H), 3.75 (m, 1H), 4.0-5.5 (m, 4H), 6.7-7.5 (m, 14H), 8.75 (br s, 2H).

37. Diphenyl (R)-1-[(S)-(tetrahydroisoquinolyl)-3-carbonyl]pyrrolidine-2-phosphonate hydro-chloride:

yield 34% (method B), hygroscopic solid, $[\alpha]_D^{20} = -71.5°$
(Cl, CHCl$_3$). ^{1}H-NMR (CDCl$_3$-CD$_3$OD/TMS): 2.0-2.7 (m, 4H),
2.8-3.2 (m, 2H), 3.5-4.5 (m, 5H), 5.1 (m, 1H), 6.9-7.6
(m, 14H).

B. AZETIDINES

1) <u>N-(N-Benzyloxycarbonyl protected amino acid)azetidine.</u>
   <u>General procedure.</u>

The appropriate N-benzyloxycarbonyl protected
amino acid (10 mmol) and N-methyl-morpholine (10 mmol, 1.01
g) in dry tetrahydrofuran (20 ml) were cooled to -15°C and
isobutyl chloroformate (10 mmol, 1.37 g) was added. After 30
minutes a solution of azetidine (10 mmol, 0.57 g) in
tetrahydrofuran (20 ml) was added to the mixed anhydride
solution and stirring was continued for 3.5 to 4 hours at
room temperature. After removing N-methylmorpholine
hydrochloride by filtration, the solvent was evaporated
under reduced pressure. The residue was dissolved in ethyl
acetate, washed with 5% citric acid solution (2x), water, 5%
sodium bicarbonate (2x) and brine. The organic layer was
dried over sodium sulphate and evaporated under reduced
pressure.
The following compounds were prepared:
N-(N-benzyloxycarbonylglycyl)azetidine. Yield: 88%. M.p.:
94-95°C.
N-(N-benzyloxycarbonyl-(S)-phenylalanyl)azetidine. Yield:
90%. Oil.
N-(N-benzyloxycarbonyl-(S)-prolyl)azetidine. Yield: 68%.
M.p.: 87.2-87.7°C.
N-(N-benzyloxycarbonyl-(S)-thioprolyl)azetidine. Yield: 76%.
M.p.: 63.1-64.1°C.

2) <u>N-Amino acid azetidine (hydrogenolysis).</u>

The protected compound (5 mmol) was dissolved in
dry methanol (20 ml). After displacing the air with
nitrogen, palladium-on-charcoal 10% (0.5 g) was added.
Nitrogen was blown over the solution for a few minutes
before introducing the hydrogen. Carbon dioxide evolution

was followed with a half-saturated and filtered barium
hydroxide solution. When the reaction was finished, the
flask was warmed in a water bath at 50°C for 30 minutes.
When the flask reached room temperature the hydrogen stream
5 was stopped. The remaining gas was displaced by nitrogen and
the palladium was removed on a celite filter and washed with
methanol (3x5 ml). The filtrate was evaporated under reduced
pressure and the remaining oil was dried in a vacuum oven
with phosphorpentoxide (P_2O_5). Thus, N-Glycylazetidine
10 (Yield: 86%) and N-(S.)-Prolylazetidine were prepared.

3) N-Amino acid azetidine (acidolysis).
         Protected azetidine (5 mmol) was dissolved in
dichloromethane (10 ml) and hydrogen bromide in acetic acid
15 30 wt% (50 mmol HBr) was added. After one hour stirring, the
mixture was evaporated under reduced pressure. Methanol was
added and evaporated again (2x). Dry ether was added and the
flask was placed at -15°C. After filtration and washing the
crystals with dry ether, they were dried on sodium hydroxide
20 under reduced pressure.
N-(S)-Phenylalanylazetidine hydrobromide. Yield: 98%. M.p.:
70.8-71.2°C
N-(S)-Thioprolylazetidine hydrobromide.

25 **C. PYRROLIDINES**
    1. Z-pro-3-fluoro-pyrrolidine (PEP inhibitor)
            The intermediate 1-[N-Benzyloxycarbonyl-L-propyl]-
3(R,S)-hydroxypyrrolidine was prepared as follows. To a
mixture of N-benzyloxycarbonyl-L-proline (1.25 g, 5 mmol),
30 (R,S)-hydroxypyrrolidine (0,44 g, 5 mmol) and benzotriazol-
1-yloxytris(dimethylamino)phosphonium hexafluorophosphonium
hexafluorophosphate (2.21 g, 5 mmol) in dimethylformamide
(20 ml) was added triethylamine (1.4 ml, 10 mmol). After
stirring at room temperature overnight, water (100 ml) was
35 added and the mixture was extracted with EtOAc (3 x 50 ml).
The combined organic layer was washed with HCl (2N, 2 x 25
ml), water (25 ml), $NaHCO_3$ (5%, 2 x 25 ml) and brine (25
ml). The organic layer was dried, evaporated and purified by

column chromatography [CH_2Cl_2;CH_2Cl_2-MeOH(98-2);CH_2Cl_2-MeOH(97-3);CH_2Cl_2-MeOH(96-4);CH_2Cl_2-MeOH(95-5)] yielding 0.56 g (1.76 mmol, 35%) of the intermediate compound as an oil. ^{1}H NMR (CDCl$_3$)δ 1.70-2.00 (m, 3H, γ-CH$_2$, ß-CH), 2.00-2.20 (m,
5 3H, ß-CH, 4-CH$_2$), 3.15-3.95 (m, 6H, 2-CH$_2$, 5-CH$_2$, δ-CH$_2$), 4.15-4.55 (m, 2H, α-CH, 3-CH), 4.95-5.20 (m, 2H, CH$_2$C$_6$H$_5$), 7.20-7.35 (m, 5H, C$_6$H$_5$).

Subsequently 1-[N-Benzyloxycarbonyl-L-propyl]-3(R,S)-fluoropyrrolidine was prepared by treating a solution
10 of 1-[N-Benzyloxycarbonyl-L-propyl]-3(R,S)-hydroxy-pyrrolidine (0.31 g, 0.97 mmol) in 1,2-dichloroethane dry (10 ml) with diethylaminosulfur trifluoride (0.26 ml, 1.94 mmol) at 0°C under nitrogen. After stirring for 2 h the reaction mixture was poured in a NaHCO$_3$ solution (5%, 100
15 ml) and stirred for 15 min. This mixture was extracted with CH$_2$Cl$_2$ (2 x 100 ml) and the combined organic layer was dried, evaporated and purified by column chromatography [CH$_2$Cl$_2$;CH$_2$Cl$_2$-MeOH(99-1);CH$_2$Cl$_2$-MeOH(98-2)] yielding 0.21 g (0.66 mmol, 68%) of the compound as a solid. The compound
20 was crystallized from EtOAc-hexane. ^{1}H NMR (CDCl$_3$)δ 1.70-2.00 (m, 3H, γ-CH$_2$, ß-CH), 2.00-2.45 (m, 3H, ß-CH, 4-CH$_2$), 3.15-4.10 (m, 6H, 2-CH$_2$, 5-CH$_2$, δ-CH$_2$), 4.10-4.35 (m, 1H, α-CH, 3-CH), 4.90-5.45 (m, 3H, CH$_2$C$_6$H$_5$), 7.20-7.35 (m, 5H, C$_6$H$_5$).

25

2. <u>Synthesis of Ile-3-fluoro-pyrrolidine</u> (DPPIV inhibitor)

The intermediate 1-[N-<u>tert</u>-Butyloxycarbonyl)-L-isoleucyl]-3(R,S)-hydroxypyrrolidine was prepared as follows. To a mixture of N-(<u>tert</u>-butyloxycarbonyl)-L-
30 isoleucine (6.94 g, 30 mmol), (R,S)-3-hydroxypyrrolidine (2.61 g, 30m mol) and benzotriazol-1-yloxytris(dimethyl-amino)phosphonium hexafluorophosphate (14.6 g, 33 mmol) in dimethylformamide (60 ml) was added triethylamine (9.2 ml, 66 mmol). After stirring at room temperature overnight,
35 water (300 ml) was added and the mixture was extracted with EtOAc (3 x 150 ml). The combined organic layer was washed with HCl (2N, 2 x 100 ml), water (100 ml), NaHCO$_3$ (5%, 2 x 100 ml) and brine (100 ml). The organic layer was dried,

evaporated and purified by column chromatography
[CH_2Cl_2;CH_2Cl_2-MeOH(98-2);CH_2Cl_2-MeOH(97-3);CH_2Cl_2-MeOH(96-4)]
yielding 5.83 g (19.43 mmol, 65%) of the intermediate
compound as a foam. ^{1}H NMR (CDCl$_3$)δ 0.89 (t, J = 7.5 Hz, 3H,
5   δ-CH$_3$,), 0.93 (d, J = 6.6 Hz, 3H, γ-CH$_3$), 1.00-1.25 (m, 1H,
γ-CH) 1.42 (s, 9H, C(CH$_3$)$_3$), 1.50-1.65 (m, 1H, γ-CH), 1.65-
1.80 (m, 1H, β-CH), 1.90-2.15 (m, 2H, 4-CH$_2$), 3.40-4.15 (m,
4H, 2-CH$_2$, 5-CH$_2$), 4.15-4.35 (m, 1H, α-CH), 4.48 (br s, 1H,
3-CH) 5.33 (d, J = 9.3 Hz) and 5.42 (d, J = 9.9 Hz) (1H,
10 NH).

Subsequently the protected compound 1-[N-(tert-
Butyloxycarbonyl)-L-isoleucyl]-3(R,S)-fluoropyrrolidine was
prepared by treating a solution of 1-[N-(tert-
butyloxycarbonyl)-L-isoleucyl]-3(R,S)-hydroxypyrrolidine
15 (0.40 g, 1.33 mmol) in 1,2-dichloroethane dry (15 ml) with
diethylaminosulfur trifluoride (0.35 ml, 2.66 mmol) at 0°C
under nitrogen. After stirring for 2 h the reaction mixture
was poured in a NaHCO$_3$ solution (5%, 100 ml) and stirred for
15 min. This mixture was extracted with CH_2Cl_2 (2 x 100 ml)
20 and the combined organic layer was dried, evaporated and
purified by column chromatography [CH_2Cl_2;CH_2Cl_2-MeOH(99-
1);CH_2Cl_2-MeOH(98-2)] yielding 0.27 g (0.89 mmol, 67%) of
the compound as an oil. ^{1}H NMR (CDCl$_3$)δ 0.85-1.00 (m, 6H, δ-
CH$_3$, γ-CH$_3$), 1.05-1.25 (m, 1H, γ-CH), 1.43 (s, 9H, C(CH$_3$)$_3$),
25 1.50-1.65 (m, 1H, γ-CH), 1.65-1.80 (m, 1H, β-CH), 1.90-2.45
(m, 2H, 4-CH$_2$), 3.40-4.10 (m, 4H, 2-CH$_2$, 5-CH$_2$), 4.10-4.40
(m, 1H, α-CH), 5.20-5.35 (m, 1H, NH), 5.28 (dm, J = 52.2 Hz,
1H, 3-CH).

The DPPIV inhibitor 1-(L-Isoleucyl)-3(R,S)-
30 fluoropyrrolidine trifluoroacetate was prepared by treating
1-[N-(tert-Butyloxycarbonyl)-L-isoleucyl]-3(R,S)-
fluoropyrrolidine (0.34 g, 1.13 mmol) with TFA (5 g) for 1 h
at room temperature. The inhibitory compound was obtained in
quantitative yield after evaporation, coevaporation with
35 toluene (3 times) and MeOH (3 times). The compound was
crystallized from MeOH-Et$_2$O. ^{1}H NMR (DMSO-d$_6$) δ 0.86 (t, J =
7.2 Hz, 3H, δ-CH$_3$), 0.93 (m, 3H, γ-CH$_3$), 1.05-1.25 (m, 1H,
γ-CH), 1.40-1.65 (m, 1H, γ-CH), 1.75-1.95 (m, 1H, β-CH),

2.00-2.30 (m, 2H, 4-CH$_2$), 3.35-4.10 (m, 5H, 2-CH$_2$, 5-CH$_2$, α-
CH), 5.38 (dm, J = 52.9 Hz, 1H, 3-CH), 8.18 (s, 3H, NH$_3^+$).

D. REDUCED PEPTIDES

5   The preparation of methyl (S,S)-3-aza-2-benzyl-4-
(1-glycyl-2-pyrrolidinyl)butanoate and methyl (S,S,S)-3-aza-
2-benzyl-4-(1-phenylalanyl-2-pyrrolidinyl)butanoate was
performed as follows.
To a solution of N-t-butyloxycarbonyl-(S)-prolinal (12.35
10 mmol, 2.46 g) in dry methanol (75 ml) were added at 0°C, 7.5
g molecular sieves (3Å, powder) and (S)-phenylalanine
methylester hydrochloride (13.65 mmol, 2.94 g). Then sodium
cyanoborohydride (13.65 mmol, 0.86 g) was added and the
reaction mixture was stirred overnight at room temperature.
15 After filtration of the sieves, the solvent was evaporated
and the residue was taken up in ether. The solution was
extracted succesively with saturated sodium bicarbonate
solution and saturated citric acid solution. The combined
citric phases were alkalinized with 4 N sodium hydroxide,
20 and extracted with ethyl acetate. The latter solution was
dried over sodium sulphate and evaporated under reduced
pressure. A light yellow oil was obtained and purified by
flash chromatography on silicagel using chloroform as eluent
(64%). Deprotection was performed by dissolving this
25 derivative (2.65 mmol, 0.96 g) in trifluoroacetic acid (TFA)
(5 g). It was stirred for 1.5 hours at room temperature.
Then the TFA was evaporated and dry methanol was added and
evaporated several times to remove traces of the acid.
Addition of dry ether gave a crystalline product, which was
30 filtered and dried. (90% yield; M.p.: 97-98°C).
After coupling this compound with N-benzyloxycarbonylglycine
and N-benzyloxycarbonyl-(S)-phenylalanine, the protected
compounds were obtained. N-Benzyloxycarbonylglycine or N-
benzyloxycarbonyl-(S)-phenylalanine (2.65 mmol) was
35 dissolved in tetrahydrofuran (10 ml). After the addition of
N-methylmorpholine (2.65 mmol, 0.27 g) the solution was
cooled to -15°C and isobutyl chloroformate (2.65 mmol, 0.36
g) was added. After 1 hour, a simultaneously prepared

solution of methyl (S,S)-3-aza-2-benzyl-4-(2-pyrrolidinyl)
butaboate trifluoroacetate (2.65 mmol, 1.00 g) in
tetrahydrofuran (10 ml) was treated with N-methylmorpholine
(2.80 mmol, 0.38 g) and added to the stirring mixed

5 anhydride solution. This mixture was kept for 3 hours at
room temperature. The solvent was removed under reduced
pressure, the residue was taken up in ethyl acetate and
washed with 5% citric acid solution (2x), water, 5% sodium
bicarbonate solution (2x) and brine. After drying the

10 organic layer over sodium sulphate and evaporating the
solvent a yellow oil was obtained. The products were
purified by column chromatography on silicagel with
petroleumether:dichloromethane:ethyl acetate by gradient
elution. [Glycine analogue: 60%; Phenylalanine analogue:

15 70%].

            Deprotection of these compounds was done by
hydrogenolysis. The protected compound (1.60 mmol) was
dissolved in dry methanol (15 ml). After displacing the air
with nitrogen, palladium-on-charcoal 10% (0,40 g) was added.

20 Again nitrogen was blown over the solution before
introducing the hydrogen. The reaction was stopped when no
precipitation of barium carbonate could be detected in the
barium hydroxide solution. After displacing the remaining
gas by nitrogen, the palladium was removed on a celite

25 filter and washed with methanol (3x5 ml). The filtrate was
evaporated under reduced pressure and the remaining oil was
dried over phosphor pentoxide. The title compounds were
obtained as oils [Glycine analogue: 90%; Phenylalanine
analogue: 95%].

30

EXAMPLE 2
Evaluation of inhibitory activity

            The inhibitory activity of some of the compounds
as synthesized in Example 1 in vitro was determined by

35 evaluating the protease activity of DPPIV in the presence of
the compounds of the invention.

            All enzymatic reactions were carried out in 50 mM
Tris.HCl buffer pH 8.3 at 37°C with Gly-Pro-4-Me-2-

naphtylamine.HCl as substrate. One activity unit is defined
as the amount of enzyme which catalyzes the conversion of 1
micromole of substrate per minute at 37^{0}C. All experiments
were carried out with the same stock solution of DPPIV
5   containing 1.8 units/ml. The enzyme was diluted (usually
1/200 or 1/500) in reaction buffer immediately before the
experiment to a suitable concentration so as not to consume
more that 10% of the substrate during the incubation period.

10 b) Determination of IC$_{50}$ values

    Inhibitor solutions were made in DMSO at various
concentrations ranging between 50 and 0.01 mM. Five
microliter of the inhibitor solution (or DMSO for the
control experiment) was added to 40 microliter of DPPIV in
15 reaction buffer and incubated at room temperature during 1
hour. The remaining activity was determined by adding 5
microliter of a 1 mM solution of Gly-Pro-4-Me-2-
naphtylamine.HCl. The reaction mixture containing enzyme,
substrate and inhibitor was incubated for 20 min at 37°C.
20 The reaction was stopped by addition of 0.5 ml 100 mM
citrate pH 4.0. The fluorescence of the sample was measured
within 2 h after quenching. The measurements were done in
duplicate and corrected for background fluorescence using a
blanc with exactly the same composition except that the
25 citrate solution was added before the substrate. The IC$_{50}$
value is defined as the inhibitor concentration which causes
a 50% decrease in fluorescence.

    c) Kinetic measurements
30   The kinetics of inactivation were measured by
adding an appropriate concentration of inhibitor (0.1 or 0.5
mM) to 0.5 ml of enzyme in reaction buffer at 37°C and
determining the activity at various time points after
mixing. To achieve this, 25 microliter samples were
35 withdrawn and diluted 2-fold in 2 mM Gly-Pro-4-Me-2-
naphtylamine.HCl. The reaction was quenched with citrate
after 10 to 15 min and the fluorescence was determined. The
dilution in an excess of substrate reduced the velocity of

inhibitor binding by a factor of 20. The decrease in
fluorescence with time was fitted with the equation of a
monoexponential decay yielding the pseudo-first order rate
constant for binding (k_{obs}) in the exponent. The second order
5 rate constant (k_+) was calculated as follows: $k_+ = k_{obs}/[I]_0$
where $[I]_0$ is the concentration of inhibitor in the
incubation mixture.

d) Stability

10   Phosphonate esters form a covalent adduct with the
active site serine, analogous to the well documented
acylated enzyme intermediate which is formed when substrate
is cleaved by serine proteases. The phosphorylated enzyme is
expected to hydrolyze slowly by action of solvent or
15 nucleophilic groups on the enzyme. A stable adduct results
in stoichiometric and irreversible inhibition of DPPIV
without consumption of the inhibitor.

          The stability of the adduct was determined as
follows. DPPIV was incubated with 0.5mM inhibitor (or an
20 equivalent volume of DMSO for the control experiment) in 1
ml reaction buffer for 1 hour at 37°C, resulting in complete
inhibition of the enzyme. Excess inhibitor was removed by
gel filtration on a PD 10 column (Pharmacia Biotech,
Uppsala, Sweden) previously equilibrated with 50 mM Tris.HCl
25 buffer, pH 8.3. At regular time intervals the activity of
the adduct was compared with the control sample which
remained active for at least one month at 37°C. After 4
weeks, only a fraction of the activity was regained (about
10%) with the inhibitors mentioned in the table.

30   The following table lists the results of the above
three experiments. Compounds marked with an asterisk (*)
were racemic mixtures of diastereoisomers. The other
compounds were separated into one inhibitory and one
inactive diastereoisomer. The data in the table apply to the
35 inhibitory diastereoisomer.

Inhibitor	IC_{50} (mM)	k_+ $(M^{-1} . s^{-1})$	stability of the adduct at room temperature
Pro-Pro^P-(OPh)$_2$*	0.1	37	> 3 weeks
Phe-Pro^P-(OPh)$_2$	0.07	36	> 3 weeks
Ile-Pro^P-(OPh)$_2$*	0.35	2.3	> 3 weeks
O-tbutyl-Tyr-Pro^P-(OPh)$_2$	1.0	0.5	
pF-Phe-Pro^P-(OPh)$_2$	0.1	40	> 4 weeks
Cyclohexylala-nine-Pro^P-(OPh)$_2$	0.06	57	> 4 weeks
Ala-Pro^P-(OPh)$_2$*	2	0.7	>4 weeks
Ala-Pro^P-(OPh)$_2$*	0.5	1.7	> 4 weeks
1-naphtyl-alanine-Pro^P-(OPh)$_2$	0.3	2.3	> 4 weeks

d) Stability of selected DPPIV inhibitors in the reaction
medium and in human citrated plasma

To determine the functional stability of the
inhibitors in the reaction medium, a 10 mM stock solution in
DMSO was diluted 50-fold in 50 mM TRIS buffer pH 8.3 and
incubated at 37°C. To determine the functional stability in
plasma, a 50 mM stock solution in DMSO was diluted 50-fold
in fresh or freshly frozen citrated plasma from a healthy
donor and incubated at 37°C. At specific time intervals an
aliquot was withdrawn, added to an equal volume of DPPIV
diluted in buffer or to an equal volume of plasma and the
kinetics of inactivation were determined as described under
b). The reduction in observed pseudo-first order rate

32

constant is a measure for the amount of functional inhibitor
lost during the incubation and allowed to estimate the half-
life of the inhibitors in buffer and plasma. The following
table lists the results.

5

Inhibitor	$t\frac{1}{2}$ in buffer (h)	$t\frac{1}{2}$ in plasma (h)
(S)-Pro-(S,R)-Pro^P(OPh)$_2$	3.5 ± 0.1	4.1 ± 0.3
(S)-Phe-(R)-Pro^P(OPh)$_2$	6 ± 1	0.7 ± 0.1
(S)-Ile-(R)-Pro^P(OPh)$_2$	10 ± 5	1.4 ± 0.9
(S)-Arg-(S,R)-Pro^P(OPh)$_2$	2.6 ± 0.3	1.9 ± 0.2
(S)-pF-Phe-(R)-Pro^P(OPh)$_2$	8.9 ± 0.1	0.7 ± 0.1
(S)-Cyclohexylala-(R)-Pro^P(OPh)$_2$	4.5 ± 0.8	0.3 ± 0.1

EXAMPLE 3

Effect of DPPIV inhibitors on recall antigen response of human lymphocytes

Synthetic inhibitors of DPP IV may modulate
inadequate immune responses. As an example the effect of 2
of the products of the present invention on the recall
antigen response in comparison to their effect on a mitogen
induced stimulation of human lymphocytes was determined in a
semi _in vivo_ experiment.

**a) Isolation of the CD8⁻ fraction of human peripheral blood
mononuclear cells (PBMC)**

Human PBMC were isolated from EDTA anti-coagulated
blood from healthy donors by density gradient centrifugation
on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described
elsewhere (Boyum A, 1968, Scand. J. Clin. Lab. Invest. 21,
77), and they were depleted of monocytes by a 2 h
polystyrene adherence step (37°C).
To obtain CD8⁻ T cells, the monocyte-depleted PBMC were
incubated with Dynabeads M450 CD8 (Dynal, Oslo, Norway)

according to the manufacturers™ instructions. The
negatively selected cells were used for further
investigations.

5 **b) Cell cultures**

Proliferation after recall antigen (Tetanus
Toxoid) or mitogen (pokeweed mitogen) stimulation was
assayed by [^{3}H]thymidine incorporation. Cells were cultured
in RPMI at a final concentration of 1 x 10^6 cells/ml in the
10 presence of 5% heat inactivated human AB serum. Tetanus
Toxoid and Pokeweed mitogen were used in a final dilution of
1:200. Stock solutions of synthetic DPPIV inhibitors (250
mM) were made in DMSO; a final inhibitor concentration of
100 μmole/l was used during cell culture. Cells and
15 inhibitor or control were incubated 1 h at 37°C before the
addition of stimuli and heat inactivated serum. Controls
consisted of (1) solvent or (2) an inactive diastereoisomer
of the synthetic inhibitors. Incubation was performed at 37°
C in a 5% CO_2 humidified atmosphere. After 4 days in
20 culture, the cells were pulsed with 0,4 μCi
[^{3}H]thymidine/well (Amersham, UK), for 8h. Cells were then
harvested on glass filter paper and tritium incorporation
was determined by liquid scintillation counting.

25 **c) Results**

The results of the lymphocyte stimulations are
given in figure 2. The codes AB22, AB23, and M1 are used for
the following products :

30 AB22 : inactive diastereoisomer of Phe-Pro^P-(OPh)$_2$
AB23 : Phe-Pro^P-(OPh)$_2$
M1 : Pro-Pro^P-(OPh)$_2$ (racemic mixture)

The results show the influence of DPPIV inhibitors on recall
35 antigen stimulation of human lymphocytes <u>in vitro</u> and may
indicate their utility in the modulation of inadequate
immune responses.

EXAMPLE 4

Proteolytic processing of vasostatin by DPPIV and inhibition thereof

Chromogranin A (CGA), the best known member of the
5 chromogranin/secretogranin family of acidic proteins
contains highly conserved N- and C-terminal domains. The N-
terminus contains a penultimate proline, a possible
recognition site for DPPIV. The N-terminal fragment of
chromogranin A (CGA1-76) has been named vasostatin I,
10 reflecting its inhibitory effect on tension development in
isolated blood vessels. Vasostatin has been shown to be
produced as a family of peptides by the parathyroid cells.
Recent evidence suggests that vasostatin plays an inhibitory
role in calcium homeostasis via an inhibition of parathyroid
15 hormone secretion at low plasma calcium.

In this experiment the processing of bovine
vasostatin (CGA1-76) by purified DPPIV was studied _in vitro_
as an illustration of the possible involvement of DPPIV in
peptide metabolism.

20   Purified vasostatin (dried, 3 μg) was incubated
during 44 hours at 37°C with or without DPPIV (17 μg
corresponding to 600 mU) in Tris 2 mM pH 8.0 in a final
volume of 50 μl. After incubation, the samples were applied
onto a YMC HPLC column (ODS 5 μm, 150 x 2 mm, 300 Å). The
25 elution conditions consisted of a linear gradient of 100%
buffer A (H2O containing 0.1% TFA) to 65% buffer B
(acetonitrile containing 0.1% TFA) at a flow rate of 0.3
ml/min. UV detection was performed at 214 nm. The peptide
containing fractions were subjected to electrospray/mass
30 spectrometry to measure the relative molecular masses (Mr).

The Mr obtained were as follows:

vasostatin incubated without DPPIV : 8583.9

vasostatin incubated with DPPIV : 8373.7

This Mr of the processed peptide corresponds to vasostatin
35 3-76 and proves the release of the N-terminal Leu-Pro (MR
113.1 for Leu and 97.1 for Pro respectively) from the
parental peptide.

Addition of the inhibitor Pro-Pro[P]-(OPh)$_2$ led to a substantial decrease in the enzyme activity of DPPIV.

5 **EXAMPLE 5**

Purification of dipeptidyl peptidase IV (CD26, DPPIV) by
means of immobilized adenosine deaminase

The high specific DPP IV activity in prostasomes
(Vanhoof et al., 1992) makes them a suitable source for the
10 purification of the native DPP IV/CD26. Prostasomes are
prostate-derived organelles which occur freely in human
seminal plasma. The purification method is based on the fact
that DPPIV binds adenosine deaminase (ADA).

15 **a) Materials**

The columns, chromatography media, and Low
Molecular Weight Electrophoresis Calibration markers were
obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The
mini-PROTEAN"II cell and the 'Broad range SDS-PAGE Molecular
20 Weight Standards' from Bio-Rad (Richmond, CA, USA) were
used. Gly-Pro-4-Me-2-NA,Ala-4-Me-2-NA and adenosine
deaminase (ADA) from Calf intestinal Mucosa were obtained
from Sigma Chemical Co, St.Louis, MO, USA. All buffers for
chromatography were filtered (0.22 μm) before use.
25 Colorimetry was performed on an HP UV/VIS spectrophotometer
8450, Hewlett Packard, USA. Chromatography was carried out
on a Waters 650 Advanced Protein Purification System,
Millipore Co., Milford, MA, USA. Fluorometric measurements
were done on a model RF5000 fluorometer (Shimadzu Corp.,
30 Tokyo, Japan).

b) Procedures

Enzyme assays

Dipeptidyl peptidase IV activity was determined as
35 described previously. (Scharpé et al. 1988) Hydrolysis of L-
Ala-4-Me-2-NA (2 mmole/l) in 60 mmole/l phosphate buffer, pH
7.4, was chosen for the determination of aminopeptidase M
(mAAP, EC 3.4.11.9) activity. One unit of enzyme activity

was defined as the amount of enzyme catalyzing the formation
of 1 μmole of assay-product/min under assay conditions.

Protein determination

5   The protein concentration was determined by the
micro assay procedure of the Bradford method (Bradford,
1976), based on Coomassie Brilliant Blue G-250. BSA was used
as the standard. Interfering substances were blank
corrected.

10

Isolation of human prostasomes

       Prostasomes were isolated from human seminal
plasma as follows : seminal plasma was centrifuged at 900 g
during 10 minutes in a MSE HI-spin 21 centrifuge (MSE
15 Scientific Instruments, Sussex, UK), to pellet the
spermatozoa. The resulting supernatant was centrifuged at
105 000 g during 60 min (Beckman Ultracentrifuge model L3-
50, Beckman Instruments, München, Germany) to obtain the
prostasomes and the seminal fluid. The prostasomes were
20 washed twice in 20 mmole/Tris-HCl, pH 7.4. Afterwards they
were solubilized in the same buffer containing 1 % (v/v)
Triton X-100 and centrifuged at 40 000 g (10 min) to clarify
the solution.

25

Immobilization of adenosine deaminase (ADA)

       ADA was immobilized onto cyanogen bromide
activated Sepharose 4B following the instructions of
Pharmacia, Uppsala, Sweden. 5 mg ADA was used per ml
30 Sepharose gel. ADA solution was dialyzed extensively against
coupling buffer (NaHCO$_3$ 0.1 M, NaCl 0.5 M, pH 8.3) and added
to CN-Br-activated Sepharose 4B gel after washing the gel
during 15 min with HCl (1 mM) and equilibration with
coupling buffer. After rotating during 2 hours at room
35 temperature in an end over end mixer, the gel was washed
with coupling buffer. The remaining active groups were
blocked by incubation with Tris-HCl 0.1 M, pH 8.0 overnight
at 4°C, while rotating. The ADA-Sepharose 4B gel was washed

with 3 cycles of acetate buffer (CH₃COONa 0.1 M, NaCl 0.5 M, pH 4) followed by Tris-HCl buffer (Tris 0.1 M, NaCl 0.5 M, pH 8.0). Storage at 4°C in Tris-HCl 20 mM, pH 7.4 containing 0.02 % thiomersal was possible for at least 18 months.

Purification procedure

Step 1 : Anion exchange chromatography on DEAE-Sepharose Fast Flow

The prostasome extract was subjected to ion-exchange chromatography on a column of DEAE-Sepharose Fast Flow (2.6 x 10 cm) equilibrated with 20 mM Tris-HCl, 70 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.4, at a flow rate of 2 ml/min. The column was eluted with 300 ml of a linear gradient of NaCl (70-350 mM) in the same buffer at a flow rate of 3 ml/min.

Step 2 : Affinity chromatography onto ADA-Sepharose 4B

The active fractions of the anion exchange column were pooled, dialyzed against 50 mM Tris-HCl pH 8.0, containing 150 mM NaCl and 0.1 % Triton X-100 and applied onto the ADA-Sepharose (1.5 x 3.5 cm) column equilibrated with the same buffer (flow rate 0.35 ml/min). After washing the column with 5 column volumes equilibration buffer and subsequently with an equal amount of 50 mM Tris-HCl, pH 7.4 containing 500 mM NaCl and 0.1% Triton-X 100, the adsorbed enzymes was eluted with 2 mmole/l Tris-HCl pH 8.0, containing 0.1% Triton X-100. Column washing and elution of the enzyme was carried out at a flow rate of 1 ml/min.

The results of the purification are summarized below.

	Activity mU	Yield %
Prostasome extract	17900	100
DEAE-Sepharose	12500	70
ADA-Sepharose	6900	40

This two-step purification procedure separates aminopeptida-
se M and DPP IV, which behave very similar during most
classical chromatography procedures including anion
exchange, gelfiltration and metal-chelating affinity
5 chromatography. The DPP IV/CD26 preparation obtained via
this scheme was homogeneous on silver-stained SDS-Page.
Figure 1 shows the SDS-page from the purified DPP IV and
aminopeptidase M preparation obtained from human
prostasomes. The Mr estimated from SDS-Page were 114 000 for
10 DPP IV and 129 500 for mAPP.
Purified DPP IV was stored at -70°C. The loss of enzyme
activity after 1 year is less than 10 %. At room temperature
the enzyme preparation is stable for at least 3 weeks.

15

EXAMPLE 6

In vivo inhibition of DPPIV by Pro-Pro^P-(OPh)$_2$ (AB 3)

In order to test the effect of the inhibitors in
vivo rabbits were injected with the inhibitor and their
20 DPPIV activity was tested using Gly-Pro-4-Methoxy-2-
naphtylamide as the substrate.
4 Rabbits received a single 5 ml intravenous (ear
vein) injection of physiological saline cobtaining 0
(control), 1 mg, 5 mg and 10 mg AB 3, respectively. Blood
25 samples were taken from the central ear artery 5 min before
injection and at different points of time, directly after
the injection, after 4 hrs and after 1, 2, 3, 6 and 13 days.
Counts of white and red blood cells and platelets,
serum DPPIV activity and serum bilirubine, GOT, GPT, GT,
30 LDH, creatinine, Na, K, CK and urea were evaluated. No
differences were seen in cell counts or in the biochemical
parameters except for the DPPIV enzyme activity. The animal
receiving 10 mg AB3 died after 11 days although at day 6 the
biochemical and hematological parameters were normal.
35 Figure 3 shows serum DPPIV activity expressed as %
of serum DPPIV activity 5 min before injection.

39

EXAMPLE 7
Purification of PEP

Prolyl endopeptidase was isolated and purified to
homogeneity from human peripheral blood mononuclear cells.
5 Up till now this source was not yet used for PEP isolation.

a) Materials

N-benzyloxycarbonyl-glycyl-prolyl-4-methyl
coumarinyl-7-amide (Z-Gly-Pro-MCA), and 7-amino-4-
10 methylcoumarine were obtained from Bachem Feinchemikalien
AG, bubendorf, Switzerland. N-benzyloxycarbonyl-prolyl-
prolinal (Z-Pro-Prolinal) and N-benzyloxycarbonyl-
thioprolyl-thioprolinal (Z-thipro-thioprolinal) were kindly
provided by Prof. T. Yoshimoto (University of Nagasaki,
15 Nagasaki, Japan) and Dr. K. Kato (Yakult Co., Tokyo, Japan),
respectively. Ethylenediaminetetra-acetate (EDTA),
dithiothreitol (DTT), di-isopropylfluorophosphate (DFP),
phenylmethylsulphonylfluoride (PMSF), amastatin, bestatin,
ornithine decarboxylase, ubiquitin, α1-antitrypsin, α1-
20 casein, albumin and antibodies towards albumin were
purchased from Sigma Co., St. Louis, USA. All other reagents
were of analytical grade from E. Merck, Darmstadt, Germany.
All chromatography materials were obtained from Pharmacia,
Uppsala, Sweden. The materials used for electrophoresis were
25 purchased from Bio-Rad, Richmond, USA. The complete
equipment for HPLC was from Gilson, Paris, France. The
system for amino acid sequencing, Applied Biosystems type
470 A, was obtained from Applied Biosystems, Warrington, UK

30 b) Isolation of mononuclear cells

Buffy coats were prepared from units of whole
blood collected from blood bank donors using standard
procedures. The buffy coats were diluted with an identical
volume of 0.9% (w/v) NaCl containing 0.3 % (w/v) tri-sodium-
35 citrate. This solution was further diluted with equal parts
of Plasmasteril (Fresenius, Bad Homburg, Germany), and the
mixture was left for 1-2 h at room temperature. 5 ml of cell
suspension was layered upon 3 ml of Lymphoprep (Nycomed,

Oslo, Norway) and centrifuges at 800 x g for 20 min at 20°C. The mononuclear cells were concentrated on the gradient layer and aspirated using a Pasteur pipette. The mononuclear cell layer was washed with phosphate buffered saline (NaCl

5 8.0 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l; pH 7.4; PBS) containing 0.3% (w/v) <u>tri</u>-sodium-citrate and subsequently centrifuged at 700 x g for 10 min whereafter the pellet is redissolved in PBS containing 0.3% (w/v) <u>tri</u>-sodium-citrate. This procedure was repeated twice. The final

10 pellet was stored at -80°C.

c) Purification of prolyl oligopeptidase from mononuclear cells

      Mononuclear blood cells were disrupted by freezing

15 and thawing (-80°C and +20°C) followed by shearing in a Potter-Elvehjem at 500 rpm (2 x 15 sec). All further procedures were performed at 4°C except otherwise stated. The intact cells were removed by centrifugation at 29 000 x g for 60 min. Under constant stirring cooled acetone (-15°C)

20 was slowly added to the supernatant to a final concentration of 32%. The precipitate was removed by centrifugation in closed polycarbonate tubes at 4 500 x g for 20 min. The supernatant was treated with ammonium sulphate. Solid ammonium sulphate was added slowly and under constant

25 stirring to a final concentration of 65% (w/v). The mixture was centrifuged at 4 500 x g for 20 min. The precipitated proteins accumulated at the interphase between the acetone layer and the ammonium sulphate solution. The liquid phases were removed and the precipitate was extracted repeatedly

30 with a small amount of Tris-HCl 20 mM pH 7.5 containing 1 mM EDTA, 1 mM NaN_3 and 1 mM DTT (buffer A). After centrifugation at 4 500 x g for 10 min the supernatant was dialyzed overnight against buffer A. Before chromatography, the dialysate was centrifuged at 15 000 x g for 30 min.

35 Step 1: the supernatant was loaded on a DEAE-Sepharose fast flow column that was washed with buffer A. The flow used for loading was 1 ml/min and increased to 2 ml/min as the

41

gradient is started. The proteins are desorbed using a
linear gradient 0 to 0.25 M NaCl in buffer A.

Step 2: The active fractions from the previous step are
combined and diluted 5 times with buffer A and applied onto
5   a Q-Sepharose HiLoad column that was equilibrated with
buffer A. The flow conditions are identical as for the
previous step. The proteins are eluted with a linear NaCl
Gradient between 0-0.4 M in buffer A.

Step 3: The active fractions are combined and saturated with
10   solid ammonium sulphate to 25% (w/v). The enzyme solution
was then applied on a Phenyl Sepharose CL-6B column that was
previously equilibrated with buffer A containing 25% (w/v)
ammonium phase. The flow rate was 0.35 ml/min during loading
and elution. The proteins were eluted using a linear
15   gradient of ethylene glycol (50% (w/v) in buffer A). The
active fractions were collected and the ethylene glycol was
removed by gel filtration on a sephadex G-25 column, which
was washed with buffer A.

Step 4: Finally the enzyme was passed through a column with
20   immobilized anti-albumin antibodies at a flow rate of 0.1
ml/min.

c) Characterization

Amino acid sequencing

25   Purified enzyme was subjected to partial amino
hydrolysis in 1% (v/v) formic acid at 110°C for 4 hours. The
peptides generated were separated on HPLC using a linear
gradient between 10 and 75% (v/v) acetonitrile in H_2O
containing 0.85% (v/v) TFA. Separate peaks were vacuum dried
30   in a Savant and stored until application in the automated
sequencer at -20°C. One of the most abundant peptides was
then chosen for sequencing. The peptide was dissolved in 10%
(v/v) TFA and applied onto a polybrene membrane and
subsequently subjected to the Edman degradation.

35

Protein determination

          Protein concentrations were measured with
Coomassie Brilliant Blue G-250 according to Bradford with

BSA as the standard (Bradford, Anal. Biochem. 72, 248-254 (1976)).

Enzyme assay

5   PEP measurement was carried out as previously described (Goossens et al., Eur. J. Chem. Clin. Biochem. 30, 235-238 (1992)) with the following modifications. Incubations lasted for 20 min at 37°C and the substrate stock solution contained 5.7 mM Z-Gly-Pro-MCA dissolved in

10   DMSO. 10 μl sample was added to 100 μl K-phosphate buffer (K-phosphate 100 mM, pH 7.5, EDTA 1mM, Dtt 1 mM, NaN3 1mM), whereafter the substrate is added. The reaction is stopped by the addition of 500 μl acetic acid (1.5 M acetic acid). The fluorescence is measured at λex 370 nm and λem 440 nm,

15   slitwidth 1.5 nm on a Shimadzu RF-5000.

      Apparent K_m constants were determined by a direct linear plot using concentrations ranging from K_m/10 to K_m x 2.

Determination of pH optimum

20   The stability of PEP with respect to pH was examined by preincubating the enzyme for 150 min at 4°C at different pH values ranging from 3 to 12 after which the reaction was started under standard enzyme assay conditions.

25   The pH optimum was determined with the standard assay using Tris-acetate buffers. We investigated the pH dependence at pH values ranging from 6.2 tot 9.4.

Determination of optimal temperature

30   The stability of PEP as a function of temperature was tested by preincubating the cytosol for 20 min at different temperatures, followed by the measurement of the residual activity at 37°C. The optimum was determined by the standard enzyme assay except for the change in incubation

35   temperature, which varied from 25 to 47°C.

Determination of the iso-electric point

The iso-electric point was estimated by iso-electric focusing in free solution on an RF$_3$ (Protein Technologies, Woburn, USA). The ampholytes, range pH 4-7

5 (from the same supplier), were diluted in 20 mM Tris-HCl, pH 7.5, containing 1mM EDTA, 1 mM NaN$_3$, and 1 mM DTT and 40 % ethylene glycol. We prefocused the ampholytes for 60 min at 1600 V, after which 6 ml of the crude, dialyzed (against Tris-HCl 20 mM, pH 7.5) cytosol was injected in the

10 apparatus. The temperature never exceeded 12°C. The run was further accomplished by focusing the protein solution for 120 min at 1000 V and finally for 30 min at 500 V. All fractions were collected and both the pH and the enzymatic activity were determined in every fraction.

15

Affinity for Concanavalin A

5 ml of crude dialyzed cytosol were applied on a Concanavalin A column previously equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 M NaCl. Elution was performed

20 with the same buffer to which 400 mM methyl-α-D-mannoside had been added.

SDS-PAGE electrophoresis

Polyacrylamide gel electrophoresis was performed

25 at 200 V in 10% gels according to Laemmli. 10 % gels were manually prepared on the day of the electrophoresis, following the protocol of Bio-Rad. The running buffer consists of SDS (5 g/l), Glycine (72 g/l) and Tris (15 g/l). After the electrophoresis, the proteins were visualized

30 using silver staining.

d) Results

Prolyl endopeptidase is a cytosolic enzyme since negligible activity is found in the pellet after disruption

35 and centrifugation of the mononuclear blood cells. Additionally the enzyme elutes in one peak on both Sephacryl S-300 HR and S-100 HR, showing there is no adsorbtion to some subcellular particles and hence it is completely

44

soluble. The specific activity of PEP in mononuclear cells,
as in platelets, is 3.0 mU/mg which is high with respect to
the other human tissues that were screened for activity
(results no shown). The total activity found in mononuclear
5 blood cells and platelets is high enough to mediate the
level of prolyl oligopeptidase in human plasma. The tissue
generating serum PEP is still unknown, but since PEP is
found throughout the whole body many tissues may
participate.

10   The enzyme is stable in the pH-range 6-9 and up to
43°C. The relative molecular mass of native PEP estimated by
gelfiltration on a Sephacryl S-100 HR was 68 kDa. This was
confirmed by SDS-PAGE analysis of the purified enzyme where
one band at 76 kDa was visualized after silver staining. The
15 comparable molecular size of native and denatured enzyme
indicate the enzyme exists as a monomer. The calculated
molecular mass of 80 745 of the enzyme from its amino acid
composition further supports this result. The iso-electric
point was determined by isoelectric-focusing in free
20 solution and was 4.8. The enzyme bears no mannopyranosyl
moiety since there is no affinity for concanavaline A. The
different chromatographic separations, based on molecular
weight, hydrophobicity and charge respectively, all reveal
one peak of enzymatic activity indicating we are dealing
25 with a single enzyme capable of degrading the synthetic
substrate Z-Gly-Pro-AMC. Its molecular weight rules out any
interference by angiotensinase C, and the addition of EDTA
prevents NEP (neutral endopeptidase) to participate in the
metabolization of peptides. A variation of this purification
30 procedure was also used to purify PEP from human
thrombocytes (platelets).

EXAMPLE 8

In vitro inhibition of PEP

35   It is generally accepted that the blood pressure
is under control by the renin angiotensin system. The key
enzymes are renin and angiotensin converting enzyme.
However, it has been suggested by Goldfarb (Goldfarb, D. &

45

Novick, A. Urology 43, 572-583 (1994)) and others that other
enzymes are involved in the management of the blood
pressure. One of which is prolyl oligopeptidase. This enzyme
converts angiotensin II and angiotensin I to angiotensin (1-
5 7), degrades bradykinin and antidiuretic hormone.

In order to test the inhibition of prolyl
oligopeptidase by the compounds of the invention the
inhibitors (cfr. table) were preincubated with purified
human platelet prolyl oligopeptidase in 100 μl buffer (100
10 mM K_2HPO_4-KH_2PO_4, pH 7.5, 1 mM EDTA, 1 mM NaN_3) at 37°C during
20 min. The remaining activity was determined by adding 5 μl
substrate (Z-Gly-Pro-AMC, 5.6 mM) and measurement of the
fluoroscence at λ_{ex}370 nm, λ_{em}440 nm after a further 20 min
incubation at 37°C. The inhibitors were dissolved in
15 methanol and control experiments were performed to correct
for the presence of the solvents used. The IC_{50} value was
derived from a plot, joining the residual activity and the
dosis of the respective compounds (at least eight
concentrations tested). The K_i values were derived from a
20 direct linear plot (Dixon-plot). The measurements were
performed in triplicate and only those lines having a
correlation coefficient of at least 0.95 were used for
determination of the K_i value.

	IC_{50} (μM)	K_i (μM)
Z-Phe-azetidine	30	-
Z-Pro-azetidine	3.0	2.5
Z-thiopro-azetidine	1.8	5.0
Z-Pro-3-fluoropyrrolidine	0.45	0.10

EXAMPLE 9

Z-PEP catalyzed degradation of proline containing peptides

Several peptides (cfr. table) were incubated with
purified prolyl oligopeptidase. The compounds were dissolved
35 in H_2O. The IC_{50} value was determined as previously described

apart from the preincubation which was deleted. In a
parallel experiment, the peptides were incubated in the same
buffer, in the presence of purified human prolyl
oligopeptidase, during at least 30 min at 37°C. The general
5 fragments were separated on HPLC. Addition of 1 μmol of Z-
thioprolyl-thioprolinal to the incubation mixture of enzyme
and peptides, completely prevented the hydrolysis of the
testpeptides.

The conditions for HPLC were as follows: Vydac C_{18}
10 (2.1 x 150 mm) flow 200μL/min, linear gradient from 0%
acetonitril (0.75% TFA in H_2O) to 70% acetonitrile (in 0.75%
TFA in H_2O) during 30 min. Detection of the fragments at 220
mm, 0.100 AUFS. The fragments were controlled by amino
sequencing. This proved they were cleaved at a postproline
15 peptide bond.

	IC_{50} (μM)
Bradykinin	7
Angiotensin II	25
Angiotensin (1-7)	750
Substance P	163

It could be demonstrated that purified prolyl
oligopeptidase hydrolyses Factor X of the clotting cascade,
25 which gives evidence for a role in hemostasis and
thrombosis. Factor X (Sigma Co.) was incubated in phosphate
buffer (same as supra) containing 1 mM DTT. The clotting
factor was incubated alone as a control for autocatalysis,
with prolyl oligopeptidase and with the enzyme in the
30 presence of an inhibitor (1μmol Z-thioprolyl-thioprolinal).
These experiments present evidence that Factor X is
hydrolysed by prolyl oligopeptidase, since autocatalysis was
ruled out and addition of a specific inhibitor for the
enzyme prevented the breakdown of the clotting factor.

47

The HPLC pattern for factor X is shown in Fig. 5, which illustrates the effect of hydrolysis of Factor X at 37°C during 16 h unless otherwise stated. (a) is pure Factor X; (b) is prolyl oligopeptidase (PEP) in the presence of Z-thiopro-thioprolinal; (c) is Factor X and PEP incubated for 60 min; (d) is Factor X and PEP; and (e) is Factor X and PEP in the presence of Z-thiopro-thioprolinal.

LEGENDS TO THE FIGURES

Fig. 1 is a schematic representation of specificity of proline cleaving enzymes.

Fig. 2 shows the influence of DPPIV inhibitors on TT (tetanus toxoid) and PWM (pokeweed mitogen) stimulation of human PBMC (Example 3).

Fig. 3 is a silver stained SDS-PAGE gel showing purified DPPIV in lane 3 in comparison with molecular weight markers in lanes 2 and 4, and purified aminopeptidase M in lane 1 (Example 5).

Fig. 4 illustrates the _in vivo_ testing of rabbits of the compound (AB3) (Example 6).

Fig. 5 illustrates the hydrolysis of factor X by human platelet prolyl oligopeptidase (Example 9).

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CLAIMS

1. Compounds having a modulating activity on
serine proteases, the compounds having the general formula:

$$Z-Xaa-Y'$$

wherein

Z may or may not be present and represents a protecting
10 group, such as benzyloxycarbonyl;

Xaa represents:

alanine, methionine, arginine, phenylalanine, aspartic acid,
proline, asparagine, serine, cysteine, threonine, glycine,
tyrosine, glutamic acid, tryptophan, glutamine, valine,
15 isoleucine, lysine, leucine, L-thioproline, L-homoproline,
L-1,2,3,4,tetrahydroisoquinoline-3-carboxylic acid (Tic), L-
2,3-dihydroindol-2-carboxylic acid, L-naphtylglycine, L-
phenylglycine, L-4-phenylproline, O-benzyl tyrosine, omega-Z
lysine, omega-acetyl lysine;
20 and Y' represents:

a)   a pyrrolidide of the general formula:

25   (II)
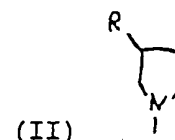
        wherein R is a halogen atom, such as Cl, F, or =O; or
b)   phosphonate or phosphonite derivatives of the general
        formula

30   (III)
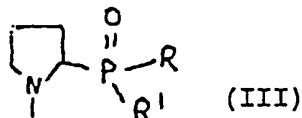
        wherein R is phenyl, R' is hydrogen, (C_1-C_4) alkyl,
        phenyl, benzyl, O-phenyl; or
35 c)   azetidines of the general formula:

  (IV)

49
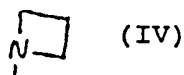
or

d)   reduced peptides of the general formula:

(V)   (VI),
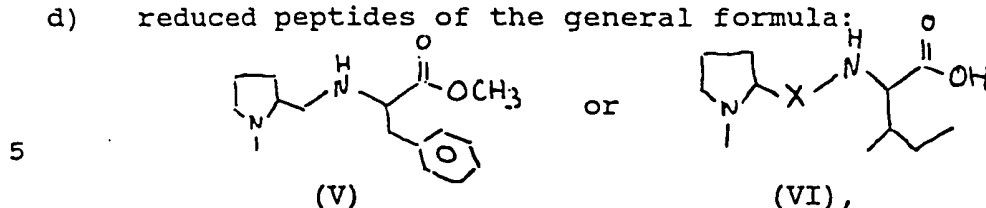
wherein X is CH_2 or $CHOH-CH_2$; or

e)   azaprolines of the general formula;

(VII)

wherein R is $CONH-(C_1-C_2)$alkyl, CONH-phenyl and o-p
substituted phenyl esters, such as p-chloro-phenyl
esters or p-benzoic acid esters, $COO-(C_1-C_4)$alkyl, COO-
phenyl, COO-benzyl, $CO-(C_1-C_4)$alkyl, CO-phenyl, CO-
benzyl, CHO or H,
or pharmaceutically acceptable salts thereof.

2. Compounds as claimed in claim 1 having an
inhibitory activity for DPPIV, in which compounds Z is not
present and the compounds further comprising a diphenyl-
phosphonate group ($^P(OPh)_2$).

3. Compounds as claimed in claim 2 selected from
the group consisting of $Pro-Pro^P-(OPh)_2$, $Phe-Pro^P-(OPh)_2$,
$Ile-Pro^P-(OPh)_2$, $O-tButyl-Tyr-Pro^P-(OPh)_2$, para-fluoro-Phe-
$Pro^P-(OPh)_2$, $Cyclohexylalanine-Pro^P-(OPh)_2$, $Ala-Pro^P-(OPh)_2$,
$Arg-Pro^P-(OPh)_2$, $1-naphtyl-alanine-Pro^P-(OPh)_2$,

4. Compounds as claimed in claim 1, having an
inhibitory activity for PEP, in which compounds Y' is a
pyrrolidine group.

5. Compounds as claimed in claim 4 which compound
is Z-Pro-3-fluoro-pyrollidide.

6. Compounds as claimed in claim 1, having an
inhibitory activity for DPPIV, in which compounds Y' is a
pyrrolidine group.

7. Compounds as claimed in claim 6 which compound
is selected from Ile-pyrrolidine-3-on and Ile-3-fluoro-
pyrrolidide.

8. Compounds as claimed in any one of the claims 1-7 for use in the treatment of inflammation, vascular diseases, auto-immune disease, multiple sclerosis, joint diseases and diseases associated with benign and malign cell
5 transformation.

9. Use of the compounds of claims 1-7 for the preparation of a medicament for treating inflammation, vascular diseases, auto-immune disease, multiple sclerosis, joint diseases and diseases associated with benign and
10 malign cell transformation.

10. Compounds as claimed in any one of the claims 1-7, optionally labelled, for use in diagnostic methods, such as fluorescence and radio assays, imaging, in situ histochemical or cytochemical staining.

15   11. Method of purifying dipeptidyl peptidase IV (DPPIV), comprising:

a) contacting a DPPIV-containing extract with a solid phase material having adenosine deaminase immobilized thereon to bind DPPIV present in the extract;
20   b) washing the material to remove unbound extract components; and

c) removal of the DPPIV from the solid phase material.

12. Method as claimed in claim 11, **characterized**
25 in that the solid phase material is Sepharose 4B and the contacting is performed on a chromatography column.

13. Method of purifying PEP, **characterized** by using human peripheral mononuclear blood cells and/or thrombocytes as a starting material.
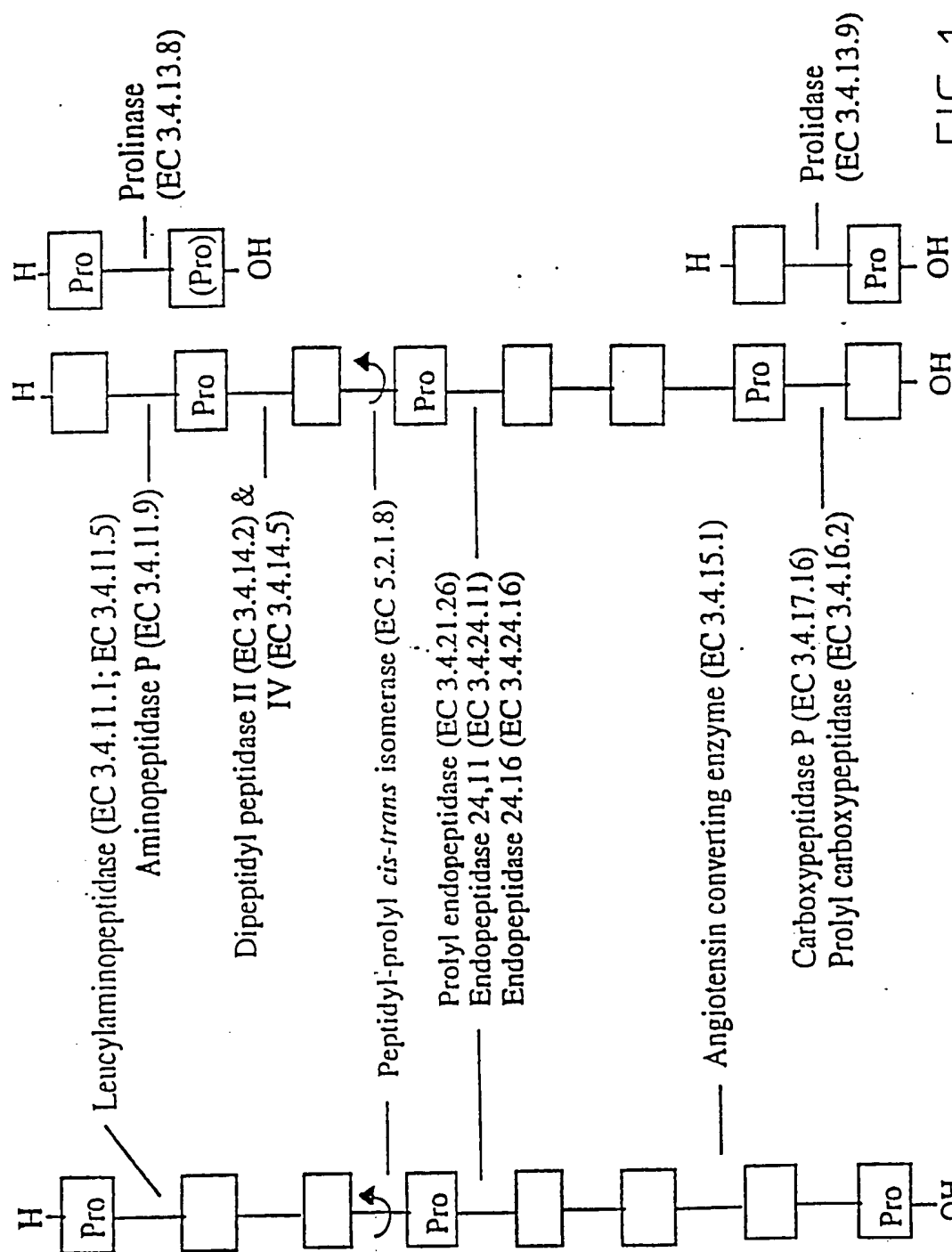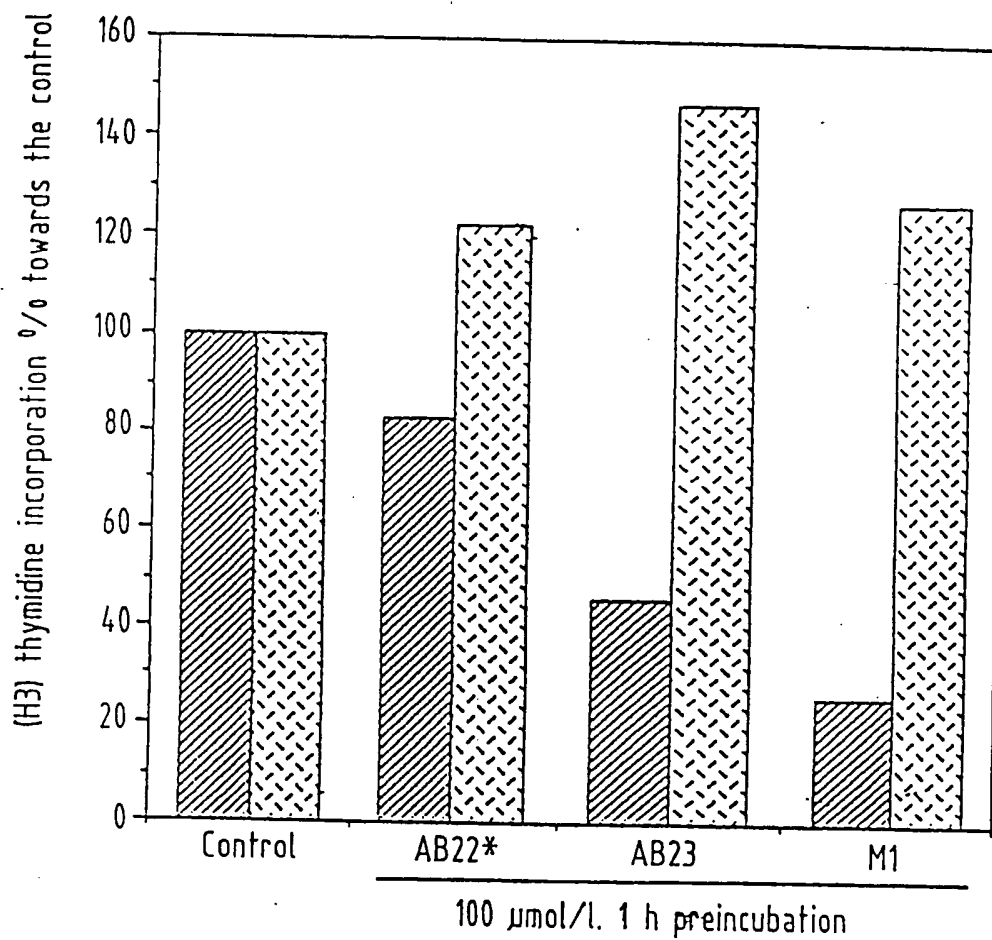
30

FIG.1

Influence of DPP I.V. inhibitors on
TT and PWM stimulation of human PBMC

100 μmol/l. 1 h preincubation

* Inactive diastereoisomer of AB23

TT

PWM

FIG. 2

FIG. 3

4/5

FIG. 4

FIG.5